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THE INITIATION OF AVIAN COCCIDIAL INFECTION WITH MEROZOITES

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The difficulty of separating *Eimeria necatrix* from *E. tenella* when the oöcysts of both species are present in the ceca, led the writer to investigate the possibility of infecting chickens with merozoites.

The only clear-cut evidence demonstrating the transmission of coccidial infection with merozoites was presented by Roudabush (1935) who infected rats with the merozoites of *E. miyairii*. Andrews' report (1927) of successful transmission of *Isospora felis* to a cat by feeding merozoites is open to some question since he did not eliminate the possibility of sporozoites being present along with the merozoites. Tyzzer (1929), despite repeated attempts, failed to infect chickens with *E. tenella* by cloacal injections of merozoites.

METHODS

The birds used in these experiments were White Leghorns from 5 to 12 weeks old that had been hatched in the laboratory and brooded in sterilized metal batteries with wire screen floors. Feed and water troughs were so arranged that fecal contamination was prevented. Stock birds to be used for experiment were placed in another room in individual steam-sterilized cages with wire screen bottoms. At least one preliminary fecal examination of each bird was made by the sugar flotation method to detect adventitious coccidial infection. From time to time the feces of stock birds and experimental birds were incubated at 30° C for 3 days in 1½ per cent potassium dichromate solution and then fed to other chickens as a further check on possible infection. Accidental infection of stock birds occurred very rarely and was quickly detected.

Coccidial infection was brought about by pipetting pure 'cultures' of sporulated oöcysts into the crop. The feces of the birds on experiment were handled with boiled instruments and sterilized glassware. All precautions were taken to prevent accidental contamination.

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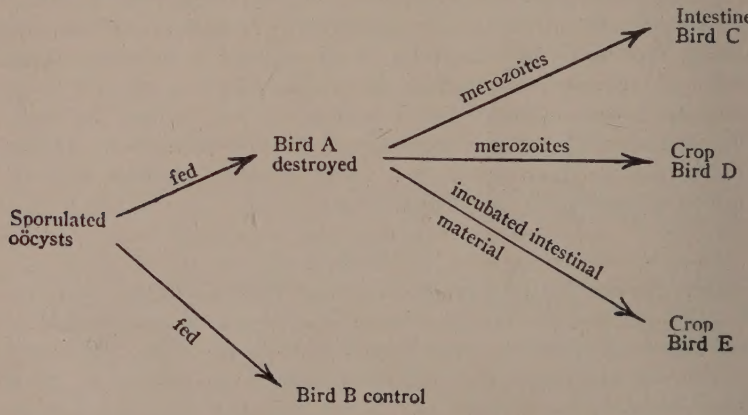
Merozoite suspensions were obtained by destroying infected birds 1 or 2 days before oöcysts were developed. The infected intestinal or cecal mucosa was scraped into 150 cc of physiological salt solution at 42° C and this suspension was promptly used for dosing the other birds. In every case microscopic examination confirmed the presence of viable merozoites. In most of the experiments 40 to 60 cc of the suspension were pipetted into the crop of one bird and an equal amount injected directly into the intestine of another. Injection into the intestine was done by passing a long, fairly pliable, hard rubber catheter, fitted with a rubber bulb, into the gizzard. Slow injection caused practically all of the suspension to go directly into the intestine. Intra-cloacal injections were done with a rubber bulb fitted to a short length of glass tubing. As much of the suspension was injected as the bird could retain. Browne (1922) has shown that fluid injected in this manner goes directly into the ceca.

The following outline indicates the plan of dosing used in most of the experiments.



Bird B was infected at the same time as A and served to indicate the time of first appearance of the oöcysts. On the day before A was to be destroyed, both A and B were transferred to clean, sterilized cages. This was done to elminate the possibility of oöcysts from the original infecting dose being found in subsequent fecal examinations. Daily fecal examinations were made of A and B thenceforth and of C and D beginning with the day they were dosed. A portion of the infected mucosa of A was incubated for 3 days in shallow layers of potassium dichromate solution and fed to bird E. Fecal examinations of E were made daily for at least 8 days to detect infection resulting from oöcysts that may have been present in A either as an adventitious infection or as a left-over of the original infecting dose.

RESULTS

The data of the first 5 experiments are recorded in Table 1.

Experiment 1 and 2. Birds A and B were infected with sporulated oöcysts of *E. maxima.* On the fourth day of infection A was destroyed and merozoites were administered to C and D as already described. Oöcysts were discharged by B on the sixth day which represents the duration of the life cycle for this species. In both experiments bird C passed oöcysts of *E. maxima* promptly on the second day after having been dosed. In the first experiment a feeding trial was necessary to detect the presence of oöcysts passed by D on the second day. In Exper. 2 the infection in D was so light that oöcysts could be demonstrated only by feeding trials. It should also be noted that in this case the infection was delayed one day. Bird E, fed incubated intestinal mucosa from A, remained free from infection in both experiments, thus indicating the absence of oöcysts from material fed C and D.

Experiment 3. E. hagani, with a life cycle of 6 days, was used in this experiment. As is shown in Table 1, both C and D passed oöcysts 2 days after they were dosed with merozoites which, in turn, had been collected on the fourth day. These oöcysts proved to be those of *E. hagani* after subsequent feeding tests and a study of their morphology had been made. Again bird E remained free from coccidial infection.

Experiment 4. Birds A and B in this experiment were infected with *E. praecox,* a species whose life cycle is completed a few hours before the fourth day. Merozoites from A were collected a few hours before the end of the third day of infection. The oöcysts discharged by C and D were those of *E. praecox* as proved by a study of their morphology and by feeding trials. Bird E remained free from coccidial infection.

Experiment 5. In the experiment with *E. necatrix* the control bird B died of acute coccidial infection on the sixth day so that the first appearance of oöcysts, which normally occurs on the seventh day, could not be recorded. The discharge of oöcysts by C and D took place two days after they had been dosed with merozoites which, in turn, had been collected on the fifth day of infection. These oöcysts, when sporulated and fed to each of two test birds, produced a fatal infection 5 days later in one and a very severe infection in the other. On examination post mortem, an extensive, severe enteritis, typical of acute infection with *E. necatrix,* was found in each bird. Bird E, again, was free from infection.

Since the oöcysts of *E. necatrix* and *E. tenella* are produced in the ceca on the seventh day of infection, ordinary means of separating these species fail. An application of the method of infection with merozoites was utilized, therefore, to obtain *E. necatrix* free from *E. tenella* when the oöcysts of both were present in the same culture. A bird fed this

TABLE 1.—*Results of fecal examinations for oöcysts (sugar flotation technique) of birds fed sporulated oöcysts and birds fed merozoites of* E. maxima, E. hagani, E. praecox, *and* E. necatrix

	Exper. 1 *E. maxima*				Exper. 2 *E. maxima*					Exper. 3 *E. hagani*			Exper. 4 *E. praecox*		Exper. 5 *E. necatrix*		
Days after dosing with sporulated oöcysts ...	4	5	6	7	4	5	6	7	8	4	5	6	3	4	5	6	7
Birds A	–[c]				–[c]					[c]			[c]		[c]	[d]	
Birds B	–	–	+	+	–	–	+	+	+	–	–	+	–	+	–	–	
Days after dosing with merozoites	0	1	2	3	0	1	2	3	4	0	1	2	0	1	0	1	2
Birds C	–	–	+	+	–	–	+	+	+	–	–	+	–	+	–	–	+
Birds D	–	–	–[a]	+	–	–	–[b]	–[a]	–[a]	–	–	+	–	+	–	–	+

[a] Incubated feces fed to a coccidia-free bird produced a pure infection with *E. maxima*.
[b] Incubated feces failed to produce coccidial infection when fed to a susceptible bird.
[c] Destroyed for source of merozoites.
[d] Died of acute infection with *E. necatrix*.

mixed culture died toward the end of the fifth day with acute hemorrhagic typhlitis typical of infection with *E. tenella*. There also were seen numerous colonies of the schizonts of *E. necatrix* visible through the serosa of the small intestine. A suspension of the mucosa from this part of the gut, prepared while the carcass was still warm, was injected into the intestine of another bird. A heavy discharge of oöcysts occurred 2 days later. On subsequent feeding trials these oöcysts proved to be those of *E. necatrix* alone. No gross or microscopic evidence of the presence of *E. tenella* could be found even after repeated feeding trials. This result was anticipated since the major part of the asexual development of *E. necatrix* takes place in the small intestine while the entire life cycle of *E. tenella* takes place in the ceca or rarely in the mucosa of the intestine surrounding the junction with the ceca. Therefore, collection of the merozoites from the small intestine only, insured the isolation of *E. necatrix* free from *E. tenella*.

Experiment 6. An experiment similar to the others was done with *E. tenella*, a species with a 7 day cycle. An infected bird was destroyed on the fifth day of infection and suspensions of merozoites were introduced into the cloaca, crop, and intestine of birds Nos. 7, 8, and 9 respectively. The absence of oöcysts from the merozoite suspension was verified by lack of infection after feeding some of the incubated cecal material to a parasite-free bird. Fecal examinations were made daily up to and including the third day after dosing. At the same time approximately ten grams of feces from each bird were incubated for 3 days at 30° C in shallow layers covered with potassium dichromate solution. The fecal suspensions were then fed to chickens and observations were made for bloody discharges on the fifth day, a typical symptom of infection with *E. tenella*. The birds were destroyed 8 days after dosing at which time the oöcysts are present in greatest numbers in the ceca if infection has occurred. After examining the cecal mucosa for gross lesions and for oöcysts or schizonts, the mucosa and cecal contents were suspended and incubated in potassium dichromate solution. This material was fed to another bird and the procedure was continued until 3 serial transfers had been made. Previous experience had indicated that if no evidence of infection was found after the third transfer, the original material was free of oöcysts of *E. tenella*. The data of this experiment are shown in Table 2. Bird No. 7 passed large numbers of oöcysts 2 days after the injection of merozoites into the cloaca. These oöcysts, when sporulated and fed to a chicken, produced a fatal infection with *E. tenella*. The feces passed on the day of injection and on the following day were free from oöcysts as was proven by negative fecal examinations and lack of infection after serial transfer of incubated material. These negative results have added significance since they also

TABLE 2.—*Results of the administration of merozoites of* E. tenella *(collected on the fifth day of infection) into the cloaca, crop, and intestine of chickens*

Port of entry of merozoites	Days after injection of merozoites	Fecal examination for oöcysts (flotation method)	Results of feeding incubated feces to birds and subsequent serial transfers of incubated cecal mucosa and contents*		
			Transfer number		
			1	2	3
Bird No. 7 Cloaca	0	–	–	–	–
	1	–	–	–	–
	2	+	+		
	3	+	+		
Bird No. 8 Crop	0	–	–	–	–
	1	–	–	–	–
	2	–	–	–	–
	3	–	–	–	–
Bird No. 9 Intestine	1	–	–	–	–
	2	–	–	+	
	3	+	+		

* Positive results determined by bloody cecal discharges on 5th day and gross and microscopic lesions on 8th day post mortem.

demonstrate absence of adventitious infection with *E. tenella*. Bird No. 8 was completely negative. Oöcysts were found in the feces of No. 9 on the third day after infection. That they had been present on the second day and not detected on fecal examination is evident from the typical infection with *E. tenella* produced on the second transfer.

This experiment was repeated and identical results were obtained. Infection resulted only in those birds that had received merozoites in the cloaca or intestine. Merozoites when introduced into the crop produced no demonstrable infection.

DISCUSSION

From the evidence presented in the foregoing experiments, it cannot be doubted that coccidial infection was initiated with merozoites of 5 species. The facts that careful precautions were taken to insure the absence of coccidial infection in stock birds and that pure cultures of the particular species used were always recovered from merozoite infected birds, argue against the possibility that such infections were adventitious. The time required for the development of oöcysts when the infection was interrupted in one bird and transferred to another was equal to the duration of the life cycle in a single host. This not only adds further evidence to support the main thesis of this paper but also substantiates the concept that the length of the life cycle is determined not by any increased resistance of the host resulting from the infection, but by the inherent make-up of each species of coccidium.

The survival of merozoites after their passage through the highly acid contents of the crop, proventriculus, and gizzard was surprising. In some species, however, this passage was made not without some mortality or decrease in vitality. Merozoites of *E. tenella,* when introduced into the crop, failed to produce infection while those injected into the intestine survived and produced a light infection. The administration of *E. maxima* merozoites into the crop resulted in a very light infection. On the other hand, those placed in the intestine produced a much heavier infection.

The failure of Tyzzer to infect chickens by cloacal injections of merozoites of *E. tenella* cannot be explained. In the work reported here, prompt infection occurred and there was no difficulty in detecting the resultant heavy discharge of oöcysts by the flotation method or even by direct smear.

That infection with merozoites occurs under favorable conditions in the field is quite probable. Aside from fecal contamination of food and water, opportunities for this mode of infection are especially good in flocks addicted to cannibalism. Quite often a sick or dying bird is completely eviscerated by his fellows.

Merozoites that had been incubated for 3 days at 30° C in 1½ per cent potassium dichromate solution, a procedure used to sporulate oöcysts, failed to survive. This was evident since no infection could be demonstrated in any birds of the E series.

The possibility of infection with merozoites should not be overlooked by laboratory workers. Infection brought about by the feeding of freshly-passed feces containing non-sporulated oöcysts might be due not to these oöcysts but to merozoites also present in the feces.

SUMMARY

Coccidial infection was initiated by introducing merozoites of *E. maxima, E. hagani, E. praecox,* and *E. necatrix* into the crop and into the intestine of parasite-free chickens.

Merozoites of *E. tenella,* when introduced into the intestine or into the ceca by cloacal injection, also caused infection. These merozoites, when placed in the crop, failed to produce infection.

The separation, by the use of the merozoite infection technique, of *E. necatrix* from *E. tenella,* when both species are present in the same culture, is described.

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STUDIES ON BOVINE GASTRO-INTESTINAL PARASITES. II. IMMUNITY TO HOOKWORM AND NODULAR WORM INFECTION IN CALVES

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During experiments on the life cycle of the hookworm (*Bunostomum phlebotomum*) and the nodular worm (*Oesophagostomum radiatum*), nematode parasites of cattle, four calves have developed a certain amount of resistance to infection as judged by egg counts. Since it is believed that these observations will be of considerable interest to parasitologists and veterinarians, a description of the experiments is presented in the following pages.

The conditions under which the experiments were carried out were carefully controlled insofar as the parasitic infections were concerned. Only a brief description of the methods is given here, since a more complete statement is made in another paper (Mayhew, 1939).

The calves used in these experiments were obtained as soon as possible after birth and placed in specially constructed cages in the animal building belonging to the Veterinary Science Department. The cement floors of the rooms, as well as the cages, were cleaned twice a week to remove the larvae before they reached the infective stage. The feed and water were parasite-free. Each animal was allowed as much hay as it desired and was given a measured amount of a commercial calf feed each day. Under these conditions of management the control animals remained consistently free of parasites. At least one and as many as four uninoculated controls have been maintained under the same conditions as the inoculated animals at all times. In the inoculated animals, eggs have appeared in the manure and increases in egg counts have occurred at the expected intervals of time after inoculation. No inoculations were made until the animals were about three months old in order to allow ample time for maturing of any larvae present before the calves were placed under experimental conditions.

The method of fecal examination used throughout these experiments has been a cane sugar, centrifuge flotation, wire loop take-off procedure which the author has adapted to certain available equipment. The abbreviation EPG refers to eggs counted per gram of sediment weighed after centrifuging. That diarrhea does not affect the egg count is indicated by the maintenance of the same level after the diarrhea ends, as for example January 14 (Fig. 4a) and November 9 and 21 (Fig. 1). This

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is apparently due to the fact that the counts are based on grams of sediment weighed after centrifuging. It is hoped to give a detailed description of this procedure in a later report, but the author wishes to emphasize at this point that the same procedure was used throughout, and, therefore, the counts should be comparative.

LITERATURE

The literature of parasitology contains many excellent studies on immunity covering examples in almost all animal groups to which parasites belong. No attempt will be made to discuss all of these papers, but mention will be made of those that deal with subjects most closely related to the observations reported in the following pages. Those who are interested in further references and especially in a general discussion of the subject are referred to the following papers: Chandler, 1932; Kotlan, 1935; Cameron, 1935; Sandground, 1929; Culbertson, 1938; Taliaferro, 1934; and Stoll (1936).

Stoll (1929) placed two parasite-free lambs on pasture, having first given one an initial dose of infective stomach worm (*Haemonchus contortus*) larvae. The results were checked by egg counts and, in the course of time, both animals obtained a rather high degree of infection under conditions of natural reinfection. This high egg count, however, decreased very rapidly to a very low level soon after the maximum was reached. One of the animals was then placed under parasite-free conditions and given doses of infective stomach worm larvae administered at stated intervals. That further heavy infection did not occur was demonstrated by egg counts and post mortem by the finding of very few parasites.

Similar results have been demonstrated in experiments with the dog hookworm (*Ancylostoma caninum*) by McCoy (1931) and by Foster (1935). In these experiments it was found that a series of inoculations of infective larvae administered at stated intervals resulted in a gradual increase in egg output which reached a peak and then rapidly decreased to a very low level. Further inoculations failed to increase the infection even though in some instances very large doses of larvae were administered. That worms were actually eliminated rather than that they ceased to lay eggs was demonstrated by the recovery of adult worms from the feces during the period of decreasing egg count.

Sarles (1932) also demonstrated an acquired immunity to *Trichostrongylus calcaratus,* a nematode parasitic in the intestine of the rabbit. In his summary he says: "All of the rabbits were susceptible to infection at first as shown by increases in the egg counts roughly proportional to the number of larvae given, but after 6 to 8 weekly doses of larvae they became refractory to superimposed infection and finally discharged the

worms which they did harbor. This self-cure manifested itself by sharp drops in the egg counts associated with soft pellets and liquid feces containing adult worms. The rabbits as tested by a later second series of infections were found to possess a high degree of acquired resistance.

"In contrast to the sudden loss of infection from rabbits given repeated doses of larvae, the infection produced in a rabbit given a single dose of larvae showed a constant rate of loss over a period of 49 weeks, as determined by egg counts."

The work of Chandler (1932), Graham (1934), and Schwartz, Alicata and Lucker (1931) on the immunity developed against the nematode *Nippostrongylus muris,* a parasite of the rat, shows a phenomenon similar if not identical to that reported in the following pages. Initial infections in rats with this parasite resulted in a gradually increasing egg count which reached a peak and then fell sharply to a relatively low level. Following the drop in egg count the animals exhibited a marked resistance to reinfection.

An immunity has been demonstrated by Sheldon (1937) in the case of *Strongyloides ratti,* a nematode parasite of the rat. A marked resistance was acquired as a result of an existing infection as well as by serial inoculations with small doses of larvae. An almost absolute resistance was demonstrated to be acquired and retained as a result of a single previous infection which had been allowed to run its normal course and disappear. Rats were successfully immunized also by serial injections of heat-killed larvae.

EXPERIMENTAL DATA

Calf No. 89

This calf, a grade Jersey × brama heifer born on April 18, 1937, was given her first inoculation on June 15, 1937, and by additional doses of larvae the egg count was increased to an average of 55 with a daily range of between 23 and 111 EPG (Fig. 1). At the time the nodular worm larvae were expected to mature from the inoculation made on September 24, 1937 (1, Fig. 1), there developed a noticeable diarrhea with more or less mucus. The diarrhea existed from October 31 till November 9 and again from the 17th to 21st. It will be noted also that there was a distinct drop in the egg count from the former average of 55 to an average 30 with a range of between 13 and 49 EPG. Following the inoculation of March 11 (2, Fig. 1), a further drop to an average of 15 EPG was observed at approximately the time the hookworms were expected to mature. No diarrhea, however, was observed at this time. Again at the time the nodular worm larvae were expected to mature from this inoculation, there was a drop in the egg count to the average of 3 EPG associated with diarrhea from December 25 to 31 and on January 5 and 6.

On January 14, 1938, another period of diarrhea began which extended to January 21 and again between the 23rd and 25th, accompanied by a fall in egg count to an average of a fraction of an egg per gram. This very low count was maintained for a period of about two and one half months. This last drop, it will be observed, occurred at the time the hookworm larvae were expected to mature from the inoculation of December 23 (3, Fig. 1). One additional inoculation was made by mouth (4, Fig. 1) with no change in the egg count.

Partly because of lack of other quarters and partly because it was desired to test certain procedures of cleaning, the calf was kept on a cement floor from December 3 till the end of the experiment. From December 3 until January 22 the room was not cleaned and wood shavings were used for bedding (5 to 6, Fig. 1). From January 22 until the end of the experiment the room was cleaned once a week and shavings were provided only part of the time. Otherwise the feeding and care were the same as for the other calves under controlled conditions. In spite of the inoculations and unsanitary care, the egg count dropped to the very low level observed above and remained down except for a short period between March 22 and April 18. The highest count recorded for this later period was 2 EPG and by the last of April it had returned to its former low level.

During the entire period of time described above the calf remained in good physical condition. She was 6 months and 15 days old when the immunity developed. The total period of infection before the immunity developed was 3 months and 5 days, and diarrhea was observed to develop in 3 of the 4 times there was a drop in egg count.

Calf No. 91

This calf, a purebred Holstein male born September 21, 1937, first became infected from an inoculation made on February 14, 1938. As a result of five additional inoculations made between March 31 and June 23, the egg count rose to an average of 111 EPG (Fig. 3b). At approximately the time for the nodular worm larvae to mature from the inoculation of June 23 (1, Fig. 3b), a rather prolonged period of diarrhea occurred, lasting from July 28 to August 17. It will be noted, also that there was a sharp drop in the egg count from the 111 average, first to an average of 54 EPG for a period of 15 days. Subsequently a second drop occurred within 3 days to an average of 3 EPG. Examination of the daily egg count curve (Fig. 3c) shows that essentially the same level was maintained for a period of about two months with the exception of possibly a slight temporary increase September 13 and October 7.

Between October 20 and February 11, the egg count remained steadily at a very low level. That a distinct immune reaction had been estab-

lished is indicated by the fact that seven inoculations of hookworm and nodular worm larvae were made during this period as indicated (1 to 7, Fig. 3c). No additional periods of diarrhea were observed other than the one associated with the major drop in egg count described above, and the animal remained in good physical condition during the entire time. This animal was 10 months and 5 days old when the immunity first was manifested and had been infected 3 months and 26 days.

Calf No. 85

This calf, a purebred Holstein male born on August 2, 1936, was given a series of inoculations over a period of about 12 months with the resulting average egg count of 8 EPG. This average resulted from the maturing of the hookworm larvae of the October 22 inoculation (1, Fig. 4a) and was maintained for 11 days. On November 19 a period of diarrhea began and 3 days later a drop in the egg count occurred with the resulting average of 5 EPG. A second decrease in egg count to 3 EPG occurred on December 5 at the time the nodular worms of the October 22 inoculation and the hookworms of the November 16 inoculation were due to mature (1 and 2, Fig. 4a). This occurred at the end of the period of diarrhea. Diarrhea was again detected on December 13 to 15 with a drop in egg count to 1 and another rather prolonged period began on December 25 and extended to January 14. The later period is associated with the expected maturity of the nodular worms of the November 16 inoculation and the hookworms of the December 10 and 14 inoculations (2, 3 and 4, Fig. 4a). It will be noted also that there was a drop from the average egg count of 1 to 0.6 EPG. Two additional inoculations were made on January 6 and 18, 1938 (5 and 6, Fig. 4a), and two increases in egg counts occurred. The first, to 1 on January 24, is associated with maturing of nodular worm larvae of the December 14 inoculation and hookworm larvae of the January 6 inoculation. The second increase, to 3 on February 15, resulted from maturing of nodular worm larvae of January 6 inoculation.

It will be noted that a series of four inoculations were then made rather close together on February 23, 28, March 8 and 13, 1938 (7, 8, 9, and 10, Fig. 4b), and that a prolonged period of diarrhea occurred from March 12 to 31. Two increases in egg count resulted from these inoculations, one occurring about March 24 and the other about April 24, the averages being 2 and 3, respectively. It is difficult to say to which species of larvae and to which inoculation these increases are due because of the dates on which the inoculations were made.

It was discovered on May 30 that the count had dropped to the very low level of less than 0.1 EPG. No observations on the condition of the manure and no counts had been made during the preceding 20 days, as

the animal had been removed from the animal building and placed in a pasture. During the following 8½ months the calf was kept in this pasture with three other lightly infected animals and during the first 7 months of this time the count gradually increased to an average of 55 EPG with a range of between 36 and 89 EPG. On February 17, 1939, the animal was killed and numerous nodular worms and a very few stomach worms (*Haemonchus* sp.) were recovered post mortem.

A statement should be made at this point concerning the means used to identify the species involved in the experiments with the three preceding calves, Nos. 89, 91 and 85. No. 85 was inoculated with larvae cultured from natural infections. Three calves, Nos. 80, 82 and 86, were inoculated from No. 85 and at post mortem examination contained only nodular worms and hookworms. Calves Nos. 89 and 91 were inoculated with larvae cultured from No. 85 or from their own manure. The fecal examination data indicate the presence of these two species and these only in that eggs appeared in the manure and increases occurred at the time expected for these two species. The species identifications were made through the kindness of Dr. G. Dikmans, Zoological Division, Bureau of Animal Industry, U. S. Department of Agriculture.

Calf No. 90

This calf, a grade Jersey male born September 23, 1937, was given his first inoculations on July 17 and 23, 1938, which resulted in an average egg count of 2 EPG (Fig. 2). An additional inoculation (1, Fig. 2) on August 7 resulted in an average egg count of 5 EPG, with a range of between 3 and 10. A definite diarrhea was observed between November 12 and 18 and a rapid drop in the egg count to an average of 0.6 EPG within a period of five days. The diarrhea was not associated with any maturing larvae as was the case each time with calf No. 89, since the larvae in the inoculations made on September 10 and 16 matured at least 10 days previous to the date on which the diarrhea started. The drop in egg count occurred when the calf was 13 months and 20 days old, after having been infected for a period of 3 months and 15 days.

Calf No. 95 was inoculated with larvae cultured from No. 90 and at post mortem examination was found to be infected with nodular worms only. The egg count curves of both Nos. 90 and 95 indicated a pure infection of nodular worms since eggs first were recovered from the manure as egg count increases occurred between the 37th and 41st days after inoculation. If hookworm larvae had been present they should have matured by the 20th day after administering the larvae.

DISCUSSION

The results described in the preceding pages seem to follow a close parallel to the observations of the several investigators referred to in the

section on literature; in that there is an increase in the egg output with succeeding inoculations which reaches a peak, followed by a sudden decrease, usually accompanied by some evidence of intestinal irritation and followed by a period of resistance to further inoculations. While the number of experimental animals is not large, a report of the results seems appropriate when one considers the difficulties encountered in the use of the bovine host and the length of time involved in experiments of this type.

It is, of course, premature to formulate any conclusions to the numerous questions which arise from this problem. The following paragraphs contain some comments and a general summary of the data on several points of interest.

The age of host from ½ year to 1¼ years, appears to have no relationship to the initiation of the immunity reaction in the individuals studied. The various animals developed the condition at the following ages: No. 85, 15 months and 19 days; No. 89, 6 months and 14 days; No. 91, 10 months and 5 days; and No. 90, 13 months and 20 days.

The duration of the period of previous infection apparently had no bearing on the development of the state of resistance in the animals studied, since the different animals had been infected for the following intervals of time: No. 89, 3 months and 5 days; No. 90, 3 months and 15 days; No. 91, 3 months and 28 days, and No. 85, 12 months and 5 days. The beginning of the period of infection is counted as the time eggs first appeared in the feces.

Neither does the degree of infection, as indicated by the egg count, have any influence on the development of immunity as is indicated by the following average egg counts previous to the appearance of immunity: No. 90, 5; No. 85, 8; No. 89, 55 and No. 91, 111 EPG.

That the immunity reaction set up is probably only of a temporary nature is indicated by the data on No. 85. Two peaks in egg count resulted from the inoculations made after December, as shown in Figs. 4a and b, followed by drops. This in nature suggests that it might offer protection under natural conditions since neither peak was half as high as the average of 8 preceding the first drop. McCoy (1931) and Sarles and Stoll (1935) report similar observations on the dog hookworm and cat ascarid. However, the fact that the count rose later on pasture to the average of 55 questions the advisability of depending upon the reaction as a protective measure. Nos. 89 and 91 were apparently much more resistant; No. 91 especially seemed to be quite resistant to additional inoculations (Fig. 3). Individual host variations will possibly be found to enter into the problem also. In the case of No. 90, the experiment could not be continued because of lack of space.

Eleven of the thirteen drops in egg count have been accompanied by diarrhea. The drop of December 4 in No. 89 and that of October 20 in

No. 91 were not associated with diarrhea. This condition of diarrhea has been observed to vary from a distinct softening of the manure to a liquid state. Generally a more or less increased amount of mucus is present either in the shape of strings up to 6 inches long or globules up to half an inch in diameter. Blood also is often present either in the partly clotted state or almost fresh, both on the surface and mixed with the manure.

Soft feces or a condition of diarrhea was observed by the following investigators at the height of the infection and with the loss of worms: Stoll (1929) in *Haemonchus contortus* in sheep; McCoy (1931) in *Ancylostoma caninum* in the dog, and Sarles (1932) in *Trichostrongylus calcaratus* infection in the rabbit.

That the immunity is associated with the maturing of both hookworms and nodular worms is indicated by the drop in egg count occurring in five instances with hookworms and four with nodular worms. Three drops are not definitely associated with the maturing of larvae but occur shortly after a group of larvae of one species or the other has reached maturity, as for example, the drop of No. 85 occurring on November 22 (Fig. 4a). The drop in egg count that occurred about August 13 in No. 91 is apparently due to the maturing of nodular worm larvae from the inoculation of April 23 (1, Fig. 3b) since the period of diarrhea is continuous between the two drops. One decrease, that of December 5 of No. 85, may be due to maturing of either hookworm or nodular worm larvae. One decrease, that of No. 90 on November 15, occurred 2 months after the maturing of nodular worm larvae of the August 7 inoculation.

That the egg count drops are due to the loss of worms resulting from the interaction of host and parasite, rather than to their death from old age, is indicated by the recovery of from 1 to 4 nodular worms on 6 different occasions of egg count decrease. That the number was not larger is probably due to the relatively large bulk of fecal material excreted by the bovine host and the small amount actually examined. That hookworms were not recovered is possibly due to the fact that they disintegrated before reaching the exterior since their point of attachment is so far forward in the digestive tract or to the relatively small amount of fecal material carefully examined. That the life span of these parasites is obviously much longer than the egg count levels described in these experiments is indicated by the fact that in another experiment preceding the development of immunity calf No. 85 infected with both hookworms and nodular worms maintained the same level for a period of a little over three months during which time all evidence indicated the worm burden was constant. That both hookworms and nodular worms were present was indicated by certain changes in egg counts following inoculations in

Nos. 85, 91, and 92 and by the recovery of both hookworms and nodular worms from three calves Nos. 80, 82, and 86 at post mortem examination inoculated from No. 85, preceding, during and following the experiment.

That this immunity reaction occurs under the usual conditions of management is indicated by the following observation. One of the calves in the L.S.U. dairy herd was found to have the following egg counts: on December 13, 1938, 400 EPG; on December 14, 920 EPG; on December 16, 227 EPG; on January 11, 1939, 19 EPG; and on January 13, 27 EPG. The animal was in very bad condition and had a severe case of pneumonia and was destroyed. At post mortem only 60 hookworms and a very few *Cooperia* sp. were recovered, although there was much irritation and other evidence of the former presence of parasites.

The observations reported in the preceding pages may be of some interest to veterinarians in that they emphasize the importance of a correct method of fecal examination before treatment, since the administration of anthelmintics would be unnecessary if the animal had already lost the major part of its parasites. At the same time the writer wishes to emphasize again the limitations of any of the methods employed at the present time when applied to the bovine host by the veterinarian.

Our knowledge concerning the egg output of the various species and combination of species and the various factors within the host acting upon the number of eggs eliminated, is at the present time very limited. It is thus difficult to make a correct interpretation of the number of eggs in terms of parasites present and damage to the host.

SUMMARY

Immunity to a mixed infection of nodular worms and hookworms induced by previous infection is described in three calves. Immunity to a pure infection of nodular worms is described in one calf.

The development of the immune reaction was not associated with age of host (range $\frac{1}{2}$ to $1\frac{1}{4}$ years), length of the infection period, or the degree of infection in the experiments described.

Three of the animals were resistant to reinfection as tested by reinoculation over a period of 6 months, although one was somewhat less so than the others. That the resistance is probably not permanent, however, is indicated by the fact that one individual acquired a much higher infection after 5 months under non-experimental conditions than under the controlled conditions. The resistance of the fourth was not tested.

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Explanation of Plates

Inoculations are indicated by a short vertical line at the beginning of a horizontal line. The next short vertical line indicates the end of 20 days and the one at the end of the line 40 days after inoculation.

Averages of egg-counts for the various periods of time are indicated by heavy transverse lines and the figures at the end of the line are the average for the period.

Dir. Days on which diarrhea was observed are indicated by short vertical lines extending upward from the horizontal.

Plate I

Fig. 1. Graph of eggs counted per gram for calf No. 89. The end of the 40 day period only is indicated in inoculation 1. In inoculation 3 the first period indicated is 14 days and the second is 40 days. During the time indicated between No. 5 and No. 6 the calf was kept on a concrete floor with shavings for bedding, and the bedding was not changed during the period. After January 21, indicated at No. 6, no bedding was used part of the time while shavings were provided part of the time and changed once a week. The graph starts in October, 1937.

Fig. 2. Graph of eggs counted per gram for calf No. 90. Graph starts in July, 1938.

Plate II

Fig. 3. Graph of eggs counted per gram for calf No. 91. It should be noted that Fig. 3a precedes Fig. 3b, the egg-counts running from 0 to 16 in 3a and from 16 upward in 3b. Also Fig. 3c follows 3b, the dates being consecutive but the counts in 3c range from 0 to 16 while the lowest on 3b is 16. Graph starts in March, 1938.

Fig. 4. Graph of eggs counted per gram for calf No. 85. Fig. 4a, it will be noted, precedes 4b. Graph starts in October, 1937.

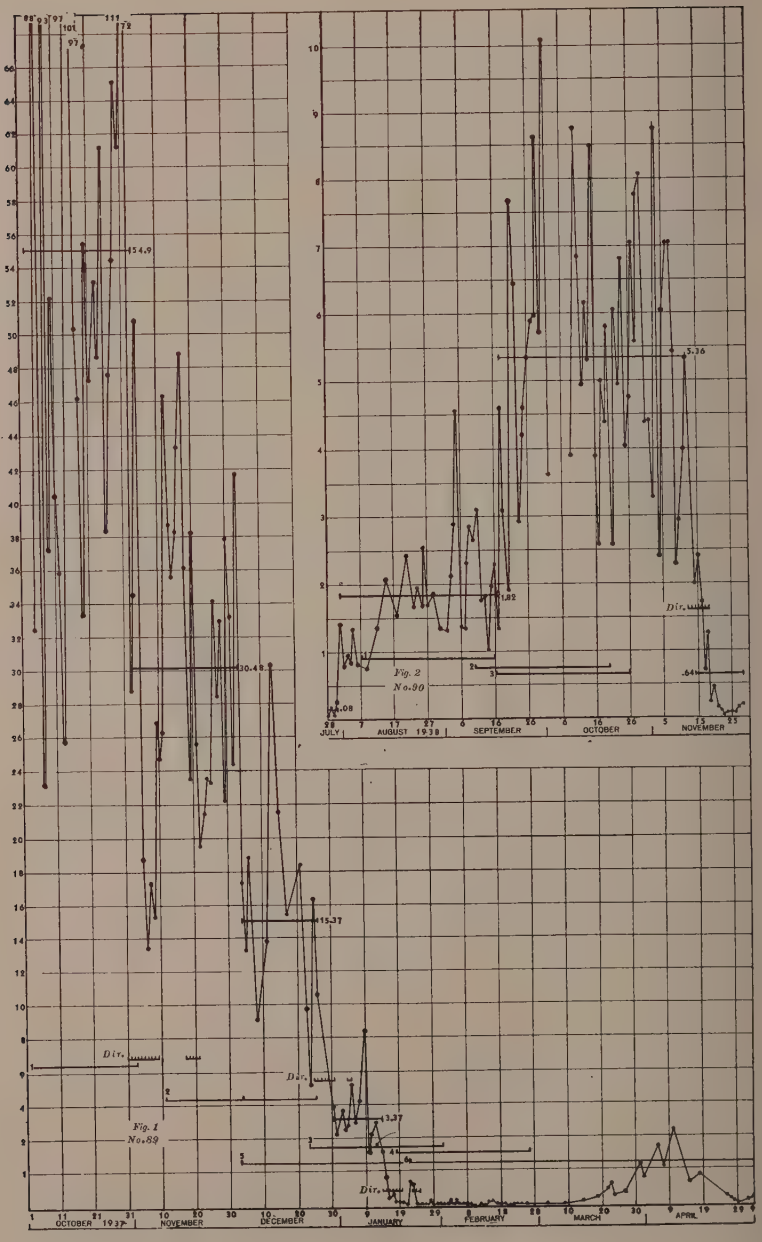
Fig. 1
No. 89
Fig. 2
No. 90
OCTOBER 1937
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DECEMBER
JANUARY
FEBRUARY
MARCH
APRIL
JULY
AUGUST 1938
SEPTEMBER
OCTOBER
NOVEMBER
Dir.
154.9
30.48
15.37
13.37
5.36
1.82
.64

PLATE I

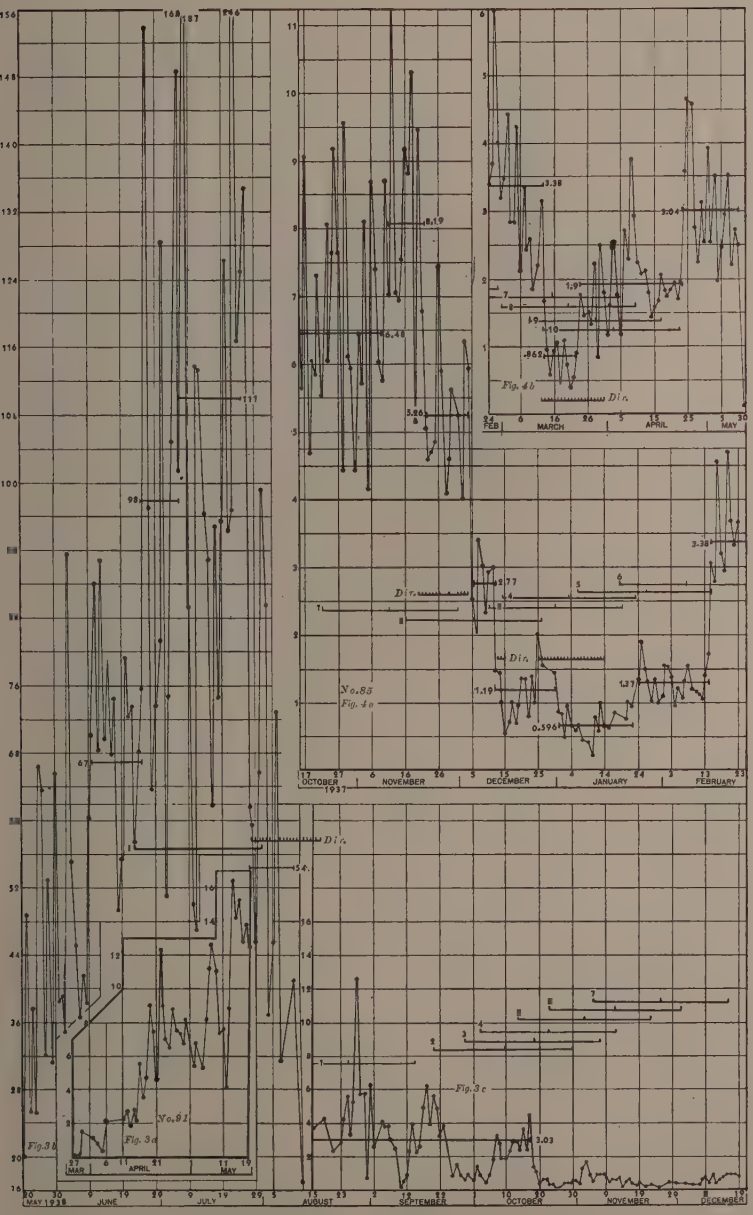
Fig. 4b
Dir.
Fig. 4c
No. 83
Dir.
Fig. 3c
Fig. 3b
Fig. 3a
No. 91
Dir.
MAY 1938
JUNE
JULY
AUGUST
SEPTEMBER
OCTOBER
NOVEMBER
DECEMBER
FEB
MARCH
APRIL
MAY
OCTOBER 1937
NOVEMBER
DECEMBER
JANUARY
FEBRUARY

PLATE II

EXCYSTMENT AND STERILIZATION OF METACERCARIAE OF THE AVIAN STRIGEID TREMATODE, *POSTHODIPLOSTOMUM MINIMUM*, AND THEIR DEVELOPMENT INTO ADULT WORMS IN STERILE CULTURES

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In a recent communication (Ferguson, 1939) the results of experiments performed in an attempt to grow in sterile cultures the adult stage of a strigeid, *Posthodiplostomum minimum* (MacCallum, 1921), were briefly outlined. The present paper will show that, with this trematode of warm-blooded vertebrates, growth and tissue differentiation, resulting in the development of adult worms producing eggs and spermatozoa, have been obtained under controlled temperature conditions in artificial media free from microörganisms.

No attempt is made here to review work of the several investigators who have tried to culture in vitro parasitic worms of vertebrates. The publications of McCoy (1935), Lapage (1937), Stunkard (1937), and Hoeppli, Feng, and Chu (1938) emphasized the desirability of growing these forms in artificial media, but none of the authors reported marked growth and tissue differentiation of parasitic worms in cultures. Glaser and Stoll (1938a, 1938b), however, made definite progress in the sterile cultivation of stages in the life cycle of the sheep nematode, *Haemonchus contortus*. They were able to carry their worms in cultures from the egg through several of the regular stages in development and to demonstrate growth and tissue differentiation. Using artificial media, Ackert, Todd, and Tanner (1938) obtained an increase in length in individuals of the fowl nematode, *Ascaridia lineata*, which had already begun their parasitic development in the intestine of chickens. Callot (1939) reported some development of the reproductive organs of the strigeid *Posthodiplostomum cuticola* when excysted metacercariae were incubated for some days in a physiological salt solution. Recently, Stunkard (1940) has referred to experiments in which metacercariae of *Plagitura parva* (adult stage in newts) were reared to sexual maturity in a bacteria-free artificial medium containing living yeast cells. However, details of the experiments and results are lacking.

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At least three species of snails of the genus *Physa,* over twenty species of fish, several members of the heron family, and very young and previously unfed chicks serve as hosts for developmental stages of *P. minimum* (Ferguson, 1936, 1937; Hunter, 1936, 1937). This evident lack of marked host specificity suggested that this worm might be more readily cultured in vitro than a form with stricter host limitations. The metacercariae of *P. minimum* develop into egg-producing adults in the intestine of the heron or chick in 35–40 hours, another factor favoring the use of this material for culture work.

Sterility was found to be necessary for the culture of *P. minimum.* Any microörganisms present, in the media or introduced with worms, multiply rapidly due to the nutrients and favorable temperature and within a day or two alter the environment to such an extent that all metacercariae die. Glaser and Stoll (1938b), as well as several of the authors mentioned above, have emphasized that for the successful cultivation of parasitic worms of vertebrates the media must be free from contamination.

Metacercariae of *P. minimum* are obtained from naturally infected pumpkinseed sunfish (*Lepomis gibbosus*) seined from waters about Princeton, N. J.

EXCYSTMENT OF METACERCARIAE AND THEIR SEPARATION FROM HOST TISSUE

The metacercaria of *P. minimum,* described by Agersborg (1926) and Hughes (1928), varies in length from about 1.0–1.5 mm and is similar in appearance to those strigeid metacercariae possessing a fairly large foliaceous forebody and shorter cylindrical hindbody (Fig. 2). This larval stage occurs encysted on or near the surface of such visceral organs of the fish host as the heart, liver, kidneys, ovaries, testes, mesenteries, and larger glands. Usually the liver and kidneys are most heavily infected and it is from these organs that worms for culture work are recovered. The cyst itself is a somewhat elongated, transparent capsule about 1 mm long and composed of one or two thin walls (Fig. 1).

Between 1935 and 1937 while the writer was working on the life history of *P. minimum,* it was determined that the metacercariae could be freed from their hyaline cysts in an acidified aqueous solution of crude commercial pepsin incubated at 37° C.

Employing this means of freeing the metacercariae, it is possible at any time to obtain thousands of free worms. Infected livers and kidneys are carefully, but not aseptically, removed from the sunfish and placed in large test tubes (22 mm × 180 mm) half filled with filtered pepsin solution (Berkefeld N). The tubes, first warmed under a tap, are held for one hour at a temperature of about 37° C by placing them in an incubator (37° C). This period of treatment, together with an

occasional vigorous shaking of the tubes, is usually sufficient to free all the cysts from the tissues to which they are attached and also to liberate large numbers of worms from the cysts themselves. The contents of the tubes are next emptied into a sterile Petri dish and as many pieces of tissue as possible removed with sterile forceps and discarded. By means of sterile pipettes the material is then transferred successively to 6 large test tubes half filled with an autoclaved three-salt Ringer's solution (Parker, 1939). In this way most of the finer debris is removed. Next the worms, both free and still encysted, are transferred with the remaining tissue fragments to a tube of the sterile pepsin solution. This tube is warmed to about 37° C and placed in the incubator for 20–30 minutes after which all the live worms are usually excysted. When the metacercariae are warmed, as in the pepsin solution, they become very active. It is this activity, together with the action of the digestive enzyme on the cyst wall, which eventually frees the worm.

The metacercariae are now transferred by pipette through several tubes of the sterile Ringer's until very little suspended matter remains in the liquid above the worms which have settled to the bottom. They may then be removed, a few hundred at a time, to a sterile Petri dish containing warm Ringer's solution. By gently rotating the dish all worms can be brought to the center. Activated by the warm Ringer's many of them swim from the center, leaving the tissue debris behind. Under a dissecting microscope a few worms at a time can then be drawn up in a fine pipette and transferred to a test tube (18 mm × 160 mm) of sterile Ringer's. This operation is repeated, if necessary with the aid of a sterile dissecting needle to isolate particular worms, until all live metacercariae have been removed from the dish.

During the above procedure, which requires about 3 hours, rigid attention is not paid to methods for ensuring sterility, since livers and kidneys of the fish are not removed aseptically and several operations must be carried on in open containers. Although it has been found that this passage of the metacercariae through several tubes of sterile Ringer's and the removal of all visible debris leaves the metacercariae fairly free from contamination, further sterilization measures must be taken before bacteria-free material can be obtained with regularity.

STERILIZATION OF METACERCARIAE

The simple method employed for removing microörganisms associated with the excysted metacercariae is somewhat similar to that developed by Cleveland (1928) and Glaser and Stoll (1940) for obtaining bacteria-free protozoa and nematode larvae, respectively. However, no chemical solutions such as those used by Glaser and Stoll are necessary. The metacercariae, being rather large, fall rapidly through a

column of liquid and leave the small, lighter bacteria behind. By taking advantage of this rapid settling 1000 to 1500 metacercariae can be handled and regularly obtained free from bacteria in about half an hour.

The metacercariae which have been isolated from tissue debris, as outlined in the previous section, are drawn into a sterile pipette and introduced into a tube (18 mm × 160 mm) half full of the sterile Ringer's. As soon as the worms have settled to the bottom all but about 2 cm of the salt solution is rapidly drawn from the test tube with a large sterile pipette. (One of about 15 cc capacity fitted with rubber tubing so as to employ mouth suction is preferred.) From the small amount of remaining solution the worms are then transferred in a sterile pipette to another tube containing 10–15 cc of the salt solution and again allowed to settle. By repeating this process about 15 times, and employing bacteriological precautions, metacercariae free from contamination can be regularly obtained.

Samples of washed worms are inoculated into routine laboratory media under aerobic and anaerobic conditions to test for bacteria. When the washing procedures have been properly executed such tests have never revealed any contaminants.

If sterility tests show that the worms have not been freed from bacteria, it has so far been found impossible to rewash them successfully. An excretion which is rather thick, and probably sticky, fills the reserve bladders of the hindbody in most metacercariae when encysted in the tissues of the fish. Most of this material is expelled when a worm is excysted and becomes active. After standing several hours in a physiological salt solution the reserve bladders in the hindbody of many worms again become filled with excretory fluid. This material, if any is expelled and becomes adherent to the outside of the worms, probably holds bacteria so that they cannot be easily washed away.

The fact that the metacercariae are encysted and not actively feeding in the fish is a determining factor in readily obtaining sterile material for cultures. The worms are probably free from bacteria both externally and internally, and derive their nourishment in solution through the cyst wall. Microörganisms separated from metacercariae during the washing process may have been in the livers or kidneys before their removal from the fish. Most likely, however, they are picked up during operations incident to the recovery of infected tissues from the host, the excystment of the worms, and their separation from tissue debris.

STORAGE OF STERILE METACERCARIAE

It is desirable to have a supply of sterile worms on hand whenever they are needed for inoculation into cultures. With this in mind, several experiments have been carried out in an effort to find a physiological saline

solution in which the worms would remain alive for the longest time when placed in the refrigerator (5–7° C).

Of the several physiological salt solutions employed to date Tyrode's solution diluted with an equal amount of distilled water has been most favorable as a storage medium. A large number of metacercariae have remained alive and infective for baby chicks when stored in this medium for one month. The addition of a small amount of chick serum to the diluted Tyrode's had no marked effect on the survival of worms in the refrigerator. The storage medium has given best results when held to a depth of about 3 cm in small cotton-plugged Erlenmeyer flasks (50 cc capacity). About 1000 sterile metacercariae are stored in each flask.

Whenever the flasks are removed from the refrigerator and the temperature of the medium is raised, the live worms once more become very active. It can then be readily determined whether or not the metacercariae are in good condition for inoculation into cultures. A day or two after newly washed worms in diluted Tyrode's are placed in the refrigerator a few of them will be contracted, opaque, and dead. This mortality continues until after about six weeks all the metacercariae are usually dead.

CULTURE MEDIA

During preliminary work sterile metacercariae were introduced into several types of ordinary liquid media and aqueous solutions of commercial liver extract. Physiological salt solutions to which varying amounts of horse serum, rabbit serum, and extracts of chicken liver, kidney, duodenum, muscle, ovary, pituitary gland, and 10-day embryo had been added were also used. In a few experiments some worms remained alive but little or no development took place during several days of incubation at a temperature of 39° C. Worms were also placed on the chorioallantoic membrane of a few 10-day chick embryos and in some the metacercariae were inoculated into the fluid below the membrane. In every case the metacercariae died without any sign of development after 2 days of incubation. Soft gels, prepared by adding a small amount of agar to physiological salt solutions, coagulated blood serum, and coagulated egg white proved to be unsatisfactory types of environment for the worms. These semi-solid media were employed with the idea that they might offer some physical resistance to the worms wriggling in the culture, or allow them to find a certain optimum level in the medium. Likewise, tubes of physiological salt solution to which shredded filter paper, ground cellophane, quartz sand, cotton wool, or pulverized glass wool had been added were unsatisfactory. Various sizes and shapes of containers including Carrel flasks, Erlenmeyer flasks, and large and small test tubes were used to hold the media during these preliminary tests.

Finally it was found that satisfactory results were being obtained by using an all-liquid medium contained in 2 cc amounts in small test tubes (13 mm × 100 mm). The tubes are plugged with cotton stoppers and, to prevent evaporation of the liquid during incubation, the ends are capped with a piece of Parafilm.

As a medium constituent Tyrode's solution is definitely superior to several other physiological salt solutions that have been tried. In early cultures, containing 2 cc of Tyrode's and 3 drops (inner diameter of pipette tip 1.5 mm) of defibrinated blood from unfed 3 day old chicks, many metacercariae (25–40 added to each tube) remained alive and active in the incubator (39° C) for about two weeks. A few worms would show evidence of some development, but usually these cultures apparently provided only a favorable survival medium. With Tyrode's alone as a medium metacercariae usually lived but two or three days. Blood from unfed baby chicks was used since *P. minimum* develops to maturity in these animals and it was thought that their blood might supply some factors favorable for development of the metacercariae in vitro. As yet, however, there is no evidence that the worms feed on whole blood or serum in the bird host. In later experiments several tubes, to which either 2 or 3 drops of a fractionally sterilized aqueous suspension (2%) of bovine ovarian extract, yeast extract, or ground heat-killed yeast were added to the Tyrode solution and chick blood, contained metacercariae showing marked development after about 10 days of incubation. A few worms became adults and eggs and spermatozoa were formed.

Eventually it was determined that more metacercariae survived and developed into adult worms in cultures where Tyrode's diluted with distilled water, in the ratio of 5:3, was employed. Serum from unfed baby chicks was found to be a better medium constituent than defibrinated blood and, later on, favorable results were obtained with serum from chickens as old as six months. The addition of yeast extract to the mixture of diluted Tyrode's and chicken serum has proved to be more effective than the ground heat-killed yeast or suspension of bovine ovarian substance in promoting development of the metacercariae. This combination of ingredients, i.e., 2 cc of diluted Tyrode's, 3 drops of chicken serum, and 3 drops of yeast extract contained in the small test tubes, provides the best culture medium that has yet been used.[2]

In preparing the yeast extract mentioned above, living baker's yeast cells, free from bacteria, are placed on the surface of a medium composed of 1% dextrose in 2% nutrient agar contained in Blake bottles. After

[2] A dilution and particular depth of Tyrode's were used after the importance of these factors had been determined by Drs. Stoll and Glaser in their work on *Haemonchus contortus*.

about four days of growth at room temperature 10 cc of sterile tap water are added to each bottle and the yeast cells washed from the surface of the medium. This suspension is transferred to test tubes where the yeast cells gradually sink to the bottom. Enough sterile melted vaseline is added to each tube to form a film one-half inch thick over the yeast suspension. These tubes are placed for at least one month in the incubator (37° C). After that time the yeast cells are killed by heating the tubes for one hour in a water bath at a temperature of 75–80° C. The supernatant fluid comprises the yeast extract used.

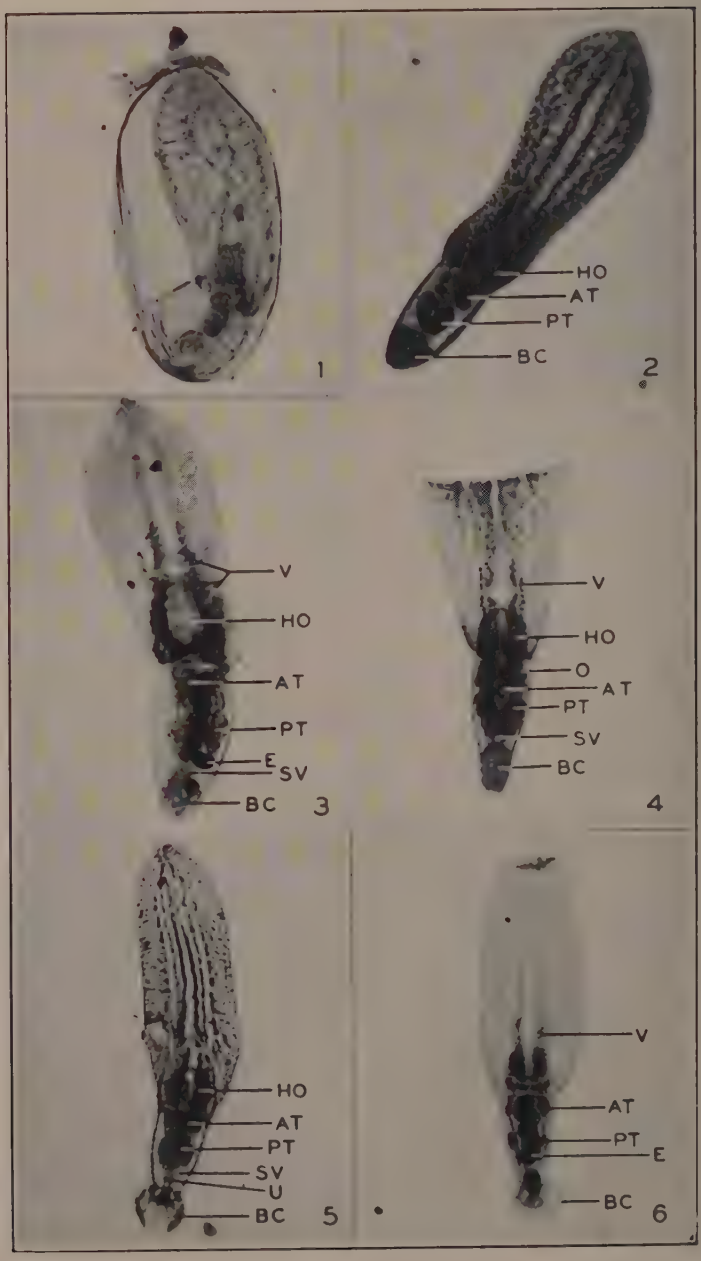
The culture medium just mentioned has a pH ranging around 6.5. It is interesting to note here that practically all adults of *P. minimum* developing in chicks are located near the middle of the duodenum, i.e., 2 or 3 inches from the gizzard. The pH of this region of the intestine in unfed baby chicks, and in fed chicks as old as 15 days, is about 6.2.

During the summer of 1939 three young black-crowned night herons (*Nycticorax nycticorax hoactli*) were taken from the nest and kept in captivity for several months. On several occasions both defibrinated blood and serum from these birds were substituted in the culture media for chicken blood and serum. In no case were adult worms obtained and in most cultures the heron blood and serum seemed to be definitely toxic. There was some evidence that blood of the herons was more favorable as a medium constituent shortly after the birds had been taken from the nest and were still young. Since these birds are natural definitive hosts of *P. minimum* their blood was expected to be more favorable for in vitro development than that of chickens. Since the herons unfortunately became infected with metacercariae of *P. minimum,* taken in with the fish diet during the first weeks of captivity, antagonistic substances or antibodies may have developed which persisted in the blood stream and were able to exert a harmful influence in cultures.

From what is now known of the most favorable medium it appears that the diluted Tyrode's and chicken serum supply conditions suitable for the survival of large numbers of metacercariae in incubated cultures and also furnish some nutritive factors which initiate development in a few worms. The yeast extract must contain factors which are responsible for the consistent growth of metacercariae into adult worms.

DEVELOPMENT IN CULTURES

Before the development obtained in cultures can be dealt with adequately it is necessary to describe briefly those changes which take place as the metacercariae of *P. minimum* develop into adult worms in the duodenum of the avian hosts, naturally in the heron and experimentally in the baby chick. The morphology of the metacercariae has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been described by Hughes (1928) and Dubois (1938) while that of the adult was given by MacCallum (1921), Noble (1936), and Dubois (1938). The adult worm is no larger than the metaceracriae and, as in some other strigeids, practically all changes during development in the bird occur within the hindbody. Here growth and differentiation normally occur in organs many of which may be observed in the metacercaria in an undeveloped condition and, together with certain secondary sexual changes, precede the production of eggs and spermatozoa, events which indicate complete sexual development. References to Figs. 2 and 3 show many of the changes taking place during normal development. In both the fore and hind body vitellaria appear (the principal change in the forebody), the bursa copulatrix is usually extruded, the testes and ovary increase in size, and the uterus and seminal vesicle make their appearance. Yolk material is transferred from the vitellaria to the vitelline reservoir before the developing adults are completely mature and later spermatozoa are observed to fill the seminal vesicle. Finally, about a day and a half after the worms enter the host, eggs are seen in the uterus. Adults of *P. minimum* produce few eggs and never have more than 8 been observed in the uterus of any worm. In chicks the greatest number of strigeid eggs are found in the feces 2–7 days after infection. Worms recovered from the duodenum of chicks two weeks after metacercariae are fed, though alive and fairly active, seldom have any eggs in the uteri, which is further evidence that only a few are produced by each individual. In herons experimentally infected with large numbers of *P. minimum* there is a similar decrease in the number of eggs in the feces after a few days. Adults developing in cultures likewise produce

Explanation of Plate

All figures refer to *Posthodiplostomum minimum*

Abbreviations

AT	anterior testis	PT	posterior testis
BC	bursa copulatrix	SV	seminal vesicle containing spermatozoa
E	egg	U	uterus
HO	holdfast organ	V	vitellaria
O	ovary	VR	vitelline reservoir

Fig. 1. Encysted metacercaria. ×50.

Fig. 2. Excysted metacercaria showing partially developed reproductive organs and inverted bursa copulatrix. ×50.

Fig. 3. Adult worm from intestine of black-crowned night heron, *Nycticorax nycticorax hoactli,* showing vitellaria, enlarged testes, spermatozoa, and eggs in uterus. ×43.2.

Fig. 4. Adult worm developed in vitro showing vitellaria, enlarged testes, ovary, and spermatozoa in seminal vesicle. ×45.

Fig. 5. Adult worm developed in vitro showing enlarged testes, spermatozoa in seminal vesicle, uterus, and extruded bursa copulatrix. ×55.3.

Fig. 6. Adult worm developed in vitro showing testes, vitellaria, eggs in uterus, and everted bursa copulatrix. ×45.

(Photographs by J. A. Carlile)

a small number of eggs. Several changes taking place in the hindbody can only be observed in sections, but those described above may be seen readily under the low power of a compound microscope, or the high power of a dissecting microscope, in the living or mounted condition.

In the early cultures in which the metacercariae lived and showed some development the testes and sometimes the ovary were observed to have increased in size. The most striking feature was usually the extrusion of the bursa copulatrix. It should be noted here that the bursae of metacercariae may occasionally be extruded when they are being excysted in warm pepsin solution. However, this is not the rule and large numbers of extruded bursae have only been observed after several hours of incubation in the more favorable culture environments. In media where more development occurred, such as in the combination of Tyrode's at regular concentration, chick blood, and ground heat-killed yeast or bovine ovarian extract, numbers of worms were noted with extruded bursae, enlarged ovaries (with numerous germ cells), enlarged testes (showing developing spermatozoa in histological sections), spermatozoa filling the seminal vesicle, vitellaria, and yolk cells in the vitelline reservoir. Worms often showed one, two, or three of these developmental changes, but only a few exhibited all (Figs. 4 and 5). Usually the time for this development to occur in the incubator at 39° C was about 10 days. In a few cases eggs were noted in the uteri of worms and in some instances they had already been expelled into the medium. From a study of these results it appeared that the less favorable environmental conditions would keep numbers of worms alive and produce preliminary changes such as the extrusion of the bursae, enlargement of testes, and production of vitelline material but that development in vitro would proceed no further. If these cultures were left in the incubator for two weeks or more, usually all the worms would contract, become opaque, die, and gradually begin to disintegrate. In some cultures, however, where little or no development took place, and which apparently were favorable for survival, the worms remained alive and active after nearly a month of incubation. It has been noted by the writer that in some chicks metacercariae survive and begin development without producing any eggs, and Ciurea (1930) reports similar findings on the development of *Posthodiplostomum cuticola* in unnatural hosts. No information is on hand concerning the length of life in the host of these worms which have not become sexually mature.

Tubes containing the most favorable culture medium, i.e., Tyrode's (5:3 dilution), chicken serum, and yeast extract, generally show several worms exhibiting many or all of the conspicuous preliminary changes while some will have continued their development further until spermatozoa and eggs are produced (Figs. 5 and 6). Eggs seldom appear at

the same time in all worms producing them in any one culture. The same observation has been made in infected chicks killed at regular intervals over a period of days after cysts of *P. minimum* had been fed. Eggs have recently been obtained in cultures only four days after the metacercariae were inoculated and the tubes placed in the incubator. Since eggs appear in the feces of chicks and herons 35–40 hours after infection with metacercariae it will be noted that development in recent culture experiments, while still slower than in the bird, is faster than during earlier experiments. In those cultures where eggs have been produced the adult worms will not remain alive in the incubator much more than two weeks. This observation coincides rather closely with those made in post mortem examinations of chicks which pass numerous eggs in the feces shortly after infection. Usually not a single living strigeid is found three weeks after infection. Adult worms recovered from the duodenum of chicks 10–15 days after infection, while alive, generally are contracted and sluggish as compared to those found 2–6 days after metacercariae are fed. Apparently the worms perish when they have produced their eggs in the abnormal host, as has also been observed by Mathias (1925) and Van Haitsma (1931) when they were able to infect domestic ducks and obtain adult strigeids naturally found in wild ducks. No definite information is at hand concerning the fate of adults of *P. minimum* in the normal host, the heron.

A variable number of metacercariae usually become opaque, contracted, and soon die in the most favorable media without any evidence of development. In other worms some of the previously mentioned preliminary changes appear and then death occurs. These observations are to be expected, since some metacercariae probably are not physiologically mature when excysted and only a fraction of the metacercariae given to an unfed baby chick survive and become adult worms.

Spermatozoa have been forced out of the seminal vesicle of worms from cultures and studied under the microscope. Their morphology corresponds closely with that of living spermatozoa of adults recovered from the duodenum of chicks. Although these germ-cells from worms developing in cultures have been observed in large numbers, up until the present time no active ones have been seen. Whether the spermatozoa are active when produced in the testes and die after reaching the seminal vesicle is not known.

In adults of *P. minimum* that have reached sexual maturity in cultures there is seldom an extensive development of vitellaria such as found in worms from the intestine of herons (Figs. 3 and 4). Adults maturing in chicks also seldom show conspicuous vitellaria. From the amount of yolk material observed in the vitelline reservoir of some worms in cultures it would appear that a great number of yolk cells may be formed in vitro,

but, as yet, nothing is known concerning how many are required for incorporation into the 4–8 eggs that the worms are believed to produce.

Eggs which have so far been obtained in cultures, although usually normal in size, are not entirely normal in shape (Figs. 7 and 8). Those

FIG. 7. Eggs of *P. minimum* from feces of an infected black-crowned night heron. Note shape, clearness of shell and germinal vesicle. ×72.

FIG. 8. Eggs of *P. minimum* from adult worms developed in vitro. Note shape, opaque appearance, and dim outline of yolk cells. ×72.

produced in early cultures were very opaque. However, eggs obtained recently have been somewhat clearer and it is possible to observe the yolk cells, normal in size, on the inside. There is a definite but thin membrane around the group of yolk cells making up the bulk of the egg, and the amber shell substance is distributed in a few small clusters at the surface rather than in a more or less coalesced layer around the entire egg. Eggs have been observed to rupture while still in the culture medium where they were produced and many, when placed in water or dilute physiological salt solution, soon burst allowing the yolk cells to flow out. These yolk cells may often be observed to have little globules of what is apparently amber shell substance on or near the surface. Stiles (1894), Goldschmidt (1909) and Kourí and Nauss (1938), after studying certain trematodes reported that they believed the yolk cells in their particular worms were the main source of shell substance for the eggs. Further study will likely show whether or not such is the case with *P. minimum*. If the shell material does come entirely from the yolk cells, worms which have developed in cultures may produce vitelline material somewhat deficient in shell substance. If the shell substance is produced in quantity by worms in cultures most of it may be retained in the yolk cells even after

the egg is formed or, if it separates out from the cells, may escape before being used in shell formation.

Reference to Fig. 7 shows that the germinal vesicle (ovum) of eggs from worms developing naturally in the heron (also in chicks) is clearly visible through the shell at one end. The yolk cells are not as conspicuous in the eggs from birds as in eggs from worms developing in cultures. To date it has been impossible to determine with certainty by microscopic examination of sectioned and unsectioned eggs whether or not a germinal vesicle is present. In a few instances, something which might represent this cell has been observed. As yet, no development of miracidia has been obtained in eggs set aside for weeks in distilled water, tap water, and diluted three-salt Ringer's. In this connection it should be pointed out that only a few eggs from adults of *P. minimum* maturing in the duodenum of chicks develop miracidia, whereas a large proportion of those from worms living in herons are viable. Perhaps there is some physiological deficiency in the eggs from chick feces, since the worms have developed in an unnatural host which may be unable to supply requirements found in herons.

SUMMARY

Observations on the morphology and natural development of stages in the life cycle of *P. minimum,* previously unpublished, are described. Methods have been found whereby the metacercariae can be recovered from the tissues of the fish host, excysted, separated from tissue debris, and freed from microörganisms. Sterile metacercariae remain alive and healthy for about one month when stored in the refrigerator in diluted Tyrode's solution. A culture medium, consisting of diluted Tyrode's, chicken serum, and yeast extract, causes metacercariae inoculated into it to develop into adult worms when incubated for several days at 39° C. Spermatozoa produced in cultures by mature worms, while regular in shape and size, are inactive. Eggs obtained in vitro have not been morphologically normal nor have they been viable.

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A NEW INTESTINAL ROUNDWORM FROM THE RUFFED GROUSE (*BONASA UMBELLUS*) IN THE UNITED STATES

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Heretofore it has been assumed that the common intestinal roundworm of the ruffed grouse, *Bonasa umbellus,* in the United States was *Ascaridia lineata.*[1] However, a recent study by the writer of specimens collected from the intestines of ruffed grouse from various localities in the Eastern and Middle Western States has shown that the ascarid usually encountered in the ruffed grouse in these regions is morphologically distinguishable from the large intestinal roundworm of the domestic fowl.

During the last few years, wildlife investigators and parasitologists have frequently reported *Ascaridia lineata* from the intestines of the eastern ruffed grouse. Cram (1926) reported *A. lineata* to be the common intestinal roundworm of this host in New England, New York, and Michigan. In connection with investigations on the relationship of diseases and parasites to the fluctuations in the numbers of ruffed grouse in New England and New York, Allen (1928), Allen and Gross (1926), and Gross (1928, 1929, 1930a, 1930b, 1931, and 1933) reported this worm as one of the commonest nematode parasites encountered. Clarke (1936), in his extensive studies on the biology and ecology of the ruffed grouse in Ontario, Canada, noted that *A. lineata*[2] was one of the principal nematode parasites of this bird host in that part of North America. Boughton (1937) stated that in Minnesota this ascarid was "more common than any other parasite found during the entire period of the survey," having found it in 37 per cent of the ruffed grouse and in 9 per cent of the sharp-tailed grouse examined. Fisher (1939) reported that *A. lineata* ranked second only to the gizzard worm, *Cheilospirura spinosa,* in numbers of infections of the ruffed grouse in Michigan.

Observations on the ascarid of the ruffed grouse by the writer were made on material collected from the intestines of the ruffed grouse in Maine, Michigan, New York, Minnesota, Wisconsin, Pennsylvania, and Massachusetts. This study showed that the ascarid in question differs from *A. lineata* of the domestic fowl chiefly in the position of the four ventral pairs of caudal papillae of the males. The first pair of papillae in

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[1] Synonym of *Ascaridia galli.* Since the term *Ascaridia lineata* has been used more frequently in the literature for the common chicken ascarid than *A. galli,* this designation is used in the present paper to avoid confusion.

[2] Tentatively identified by the present writer as *Ascaridia lineata.*

specimens from the ruffed grouse is located opposite the middle of the precloacal sucker. This pair of papillae in *A. lineata,* however, is always located anterior to the sucker. The second and third pairs of papillae in specimens from the ruffed grouse are located between the sucker and the cloaca, while in *A. lineata* the second pair of papillae is found opposite the middle of the sucker and the third pair is situated in the region between the sucker and cloaca. The fourth pair of papillae in the grouse form is internal and slightly anterior to the second lateral pair of papillae; the corresponding pair of papillae in *A. lineata* is slightly internal and just posterior to the second lateral pair.

The figure of a ventral view of the male tail of "*Ascaridia galli*" from the ruffed grouse, as given by Boughton (1937), is apparently incorrect, since a study of several specimens of ascarids collected from ruffed grouse in Minnesota, and determined by Boughton as *A. galli,* showed that the fourth ventral pair of papillae is between the first and second lateral pairs and not posterior to the second lateral pair of papillae.

Ascaridia lineata from poultry and the form from the grouse also differ somewhat in body size. The species from the ruffed grouse is stouter and only about one-half as long as the chicken ascarid. In ventral view, the body of the male of *A. lineata* is found to narrow abruptly and to a considerable degree just back of the cloaca; in the grouse form, this region narrows only slightly as compared with the former species.

On the basis of the present study, the writer is of the opinion that the ascarid commonly met with in the ruffed grouse of the Eastern and Middle Western States is a distinct species, since it differs not only from *A. lineata* but also other species of *Ascaridia* in characters which are regarded as specific.

Ascaridia bonasae n. sp.

(Figs. C and D)

Description.—Head with three well developed lips. Esophagus club-shaped, without a posterior bulb. Cephalic alae absent.

Male 19 to 35 mm long and 0.72 to 1.35 mm wide. Esophagus 1.87 to 2.77 mm long, club-shaped. Spicules equal, 1.8 to 2.7 mm long. Caudal papillae consisting of 10 pairs arranged, in general, as follows: Four pairs of ventrals and 6 pairs of laterals, the first or most anterior ventral pair being situated opposite the middle of the precloacal sucker; the second and third ventral pairs between the sucker and the cloaca, and the fourth ventral pair of papillae is between the first and second lateral pairs and in close proximity to the second lateral pair. The six lateral pairs of papillae are arranged roughly into two general groups; the first group consisting of three fairly large and widely separated papillae situated opposite the cloaca, and the second group of three smaller and less scattered papillae situated near the tip of the tail. The body posterior to the cloaca tapers gradually.

Female 30 to 50 mm long and 1 to 1.5 mm wide. Esophagus 2.8 to 3.4 mm long, club-shaped. Vulva situated near middle of body. Tail bluntly pointed.

Host: Ruffed grouse (*Bonasa umbellus*).

Location: Intestine.

Distribution: United States (Maine, Michigan, New York, Minnesota, Wisconsin, Pennsylvania, and Massachusetts); Canada (Ontario).

Specimens (type male and female): U. S. Nat. Mus. Helm. Coll. No. 43515.

Ascaridia bonasae is most closely related to *A. lineata* of the domestic fowl and *A. dissimilis* of the turkey. The following key will aid in differentiating the three species.

1. Male with first ventral pair of papilla anterior to precloacal sucker. *Ascaridia lineata*

   Male with first ventral pair of papilla opposite middle of precloacal sucker 2

2. Male with papillae of fourth ventral pair widely separated, situated close to base of those of second lateral pair .*Ascaridia bonasae* n. sp.

   Male with papillae of fourth ventral pair slightly separated, situated just posterior to cloaca ..... *Ascaridia dissimilis*

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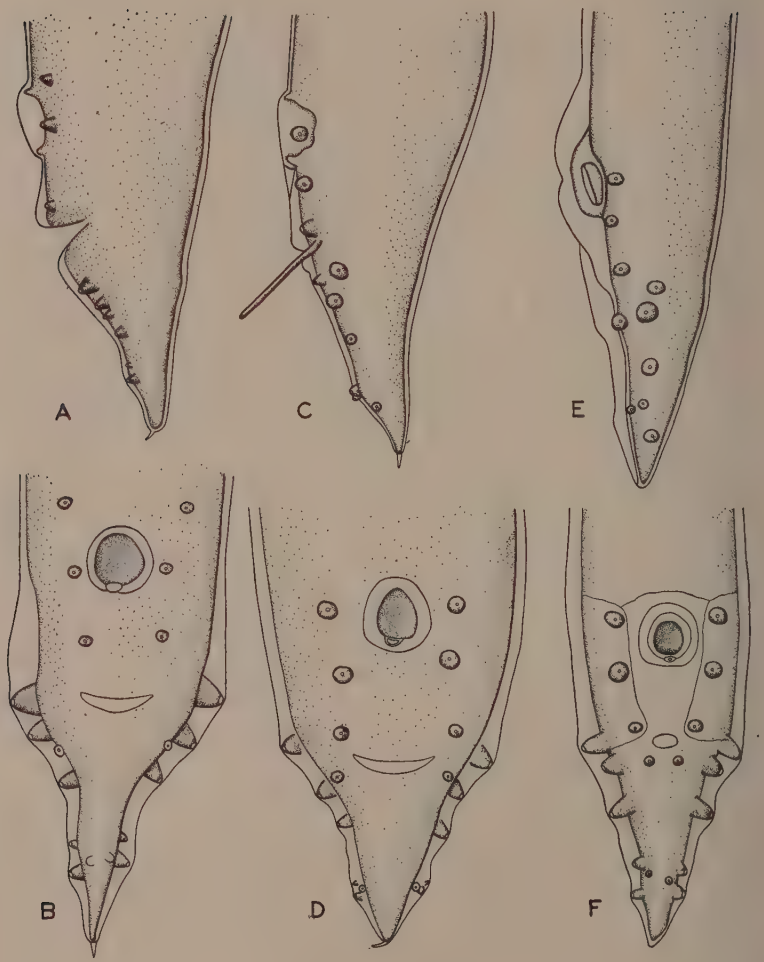

EXPLANATION OF PLATE

A. *Ascaridia lineata,* lateral view of male tail.
B. *Ascaridia lineata,* ventral view of same.
C. *Ascaridia bonasae,* lateral view of male tail.
D. *Ascaridia bonasae,* ventral view of same.
E. *Ascaridia dissimilis,* lateral view of male tail.
F. *Ascaridia dissimilis,* ventral view of same.

All drawings made to the same scale.

THE ERYTHROCYTES IN *BABESIA (PIROPLASMA) CANIS*

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Babesia canis, a hemosporidia parasitizing the erythrocytes of dogs, probably has been studied as carefully as any of the species of the family Babesidae. Since the historical and morphological aspects of this sporozoon have been covered adequately by Schilling and Meyer (1930), Wenyon (1926), Hegner (1938a) and Saunders (1937) they will not be reviewed here. A part of the symptomatology associated with infection by this blood parasite is a marked anemia which, as far as the authors are aware, has not been described in detail. In the present paper an attempt will be made to describe the anemia and the corresponding erythrocyte changes.

EXPERIMENTAL PROCEDURES

Standard hematological procedures for the enumeration of the erythrocytes, determination of the hemoglobin concentration and volume of packed red cells, were utilized and have been described elsewhere (Landsberg, 1939). The derived erythrocyte constants, the mean corpuscular volume, corpuscular hemoglobin and corpuscular hemoglobin concentration were calculated after the formula of Wintrobe. For the enumeration of the reticulated, as well as the parasitized erythrocytes, a smear was prepared by the so-called "wet method" since the parasites and reticulocytes stain equally well with brilliant cresyl blue in vital preparations. The percentages were determined by counting cells with a 1.9 mm oil objective and 10× ocular. The same preparation and magnification, utilizing a standardized ocular micrometer disc, were used to measure the erythrocyte diameters.

The original piroplasmosis strain was obtained from an animal in New York City which apparently had contracted the infection in Texas (Merenda, 1939). The experimental animals were inoculated by intravenous injection of whole blood containing the organisms, into the jugular vein about thirty days following splenectomy. For splenectomy the usual surgical procedures were carried out using ether. At all times the animals were maintained on an adequate diet under the usual laboratory conditions.

EXPERIMENTAL RESULTS

The presence or absence of the spleen has been shown to influence infection with piroplasmosis. Saunders (1937) along with others

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demonstrated that the extirpation of this organ brought about an exacerbation of the symptoms. In experimental studies the spleen has been removed, upon occasion, to assure infection; however, there appears to be no information upon the influence of splenectomy on the erythrocyte picture in piroplasmosis. Since the effect of spleen removal has been considered carefully in the present studies, the animals have been grouped on this basis (Table 1).

The physical as well as the hematological changes were essentially identical. All the dogs died as a result of infection with *Babesia canis*. There was observed a progressive decrease in weight, a loss of appetite and a general malaise. The urine of some animals was a reddish-brown and other animals showed a purulent discharge of the eyes. The temperature remained above normal with some fluctuation until a day or so before death, when a marked drop was observed. The time each animal survived after infection, as well as the maximum temperature, is given in Table 1.

In each animal an anemia was observed which increased progressively in magnitude. The presence or absence of the spleen did not appear to influence the degree of anemia since the hematological values were just as low in the animals retaining their spleens as in those from which the spleen had been removed (Table 1). The sedimentation rate was accelerated markedly. Since all the animals showed essentially the same erythrocyte change, the findings on one of them (a splenectomized animal, D252) are given in detail as a typical example (Table 2). The difference between the erythrocyte values before infection and at the time of death has been tested statistically by dividing the difference between the means by the sigma of the difference (Table 3). From Table 3 it may be observed that this is a significant difference since the value for sigma in no instance is less than ten, and any value above three is considered beyond the range of random sampling.

The corpuscular constants did not show any definite trends during the course of the infection, although in some instances they showed fluctuation about the usual range. The mean values of the constants before infection and at death were tested statistically as described above. The value of sigma (Table 3) does not exceed one in any instance, hence, there is no significant difference since such a difference may occur by random sampling.

The percentage of reticulocytes did not appear influenced either by age or splenectomy. In general it appeared that the reticulocytes were increased somewhat during the latter half of the infection; an observation consistent with the development of the anemia. In view of the recent work of Hegner (1938b), who observed that four species of avian *Plasmodium* enter the young erythrocytes of canaries and Eaton's (1934b)

TABLE 1.—*Pertinent data on 12 dogs infected with* Babesia canis

Animal No.	Sex	Wt. kg	Days until death	Temp. F° Maximum	Erythrocytes millions		Hemoglobin gm per 100 cc		Hematocrit cc per 100 cc	
					Before infection	At death	Before infection	At death	Before infection	At death
SPLEEN ABSENT										
Adults										
D216	M	...	28	105.4	6.66	1.12	11.8	1.0	50.9	5.5
D233	F	5.5	18	105.6	5.42	3.01	13.0	6.1	42.0	24.0
D251	M	9.0	19	105.0	5.27	1.78	10.0	4.0	51.2	14.0
D252	M	7.3	22	105.2	6.00	1.08	13.0	2.9	48.3	10.0
D273	M	...	10	104.1	6.36	2.05*	12.3	4.9	41.0	15.8
D280	M	11.5	23	104.6	5.48	2.83*	13.8	5.9	45.9	21.5
D305	M	12.5	16	104.1	6.14	1.73	14.1	2.7	45.0	12.0
Immature										
D224	F	...	26	104.5	5.47	0.95	13.0	1.9	43.3	7.1
SPLEEN PRESENT										
Adults										
D254	M	6.1	28	105.0	4.94	0.99	10.5	2.7	40.0	10.0
D268	M	17.4	18	105.6	6.27	3.96	13.6	6.9	45.0	33.0
Immature										
D246	F	5.0	15	105.0	5.22	1.37	7.1	3.4	37.0	11.8
D223	F	4.5	20	105.6	3.85	0.89	7.6	0.9	32.5	6.0

* Sample 3 days before death.

TABLE 2.—*The hematological findings on one splenectomized animal (D252) infected with* B. canis

Days on Experiment	R.B.C.	Hb.	Ht.	C.V.	C.H.	C.C.	Sed.	Retic.	Per cent parasitized erythrocytes		Temp.
									Single infection	Double infection	
0	5.85	13.0	48.3	83	22	27	1.0	1.2	...	...	102.4
1	Splenectomized Given 20.0 cc infected whole blood										
									0	0	103.0
2	6.00	13.5	48.0	80	22	28	0	1.8	1.8	0.6	104.0
3	...	...	...	..	..	..	..	0.8	13.0	10.6	104.6
4	...	...	...	..	..	..	..	0	0.2	0.4	102.5
5	...	...	...	..	..	..	..	0.4	1.2	0	103.8
6	...	...	...	..	..	..	..	0.8	0.4	0.2	103.6
7	4.27	9.7	35.5	83	23	27	23	0.6	0.2	0	103.2
8	...	...	...	..	..	..	..	1.0	0.4	0.2	104.0
9	...	...	...	..	..	..	..	1.4	0.2	0	104.2
10	4.48	8.9	34.0	76	20	26	26	7.4	0.6	0.2	102.0
11	...	...	...	..	..	..	..	3.0	0	0	102.6
12	...	...	...	..	..	..	..	3.6	0	0	102.2
13	...	...	...	..	..	..	..	6.4	0	0	103.6
14	4.15	8.0	32.0	77	19	25	11	9.8	0.2	0	104.8
15	...	...	...	..	..	..	..	1.6	0	0	104.6
16	...	...	...	..	..	..	..	0.2	0	0	105.2
17	3.79	7.0	30.0	79	18	23	25	1.0	0	0	104.1
18	...	...	...	..	..	..	..	1.2	0	0	103.8
19	...	...	...	..	..	..	..	0.4	3.4	3.2	104.6
20	...	...	...	..	..	..	..	1.2	1.0	0.2	104.2
21	3.03	6.4	20.0	67	21	32	30	1.2	13.6	9.6	103.2
22	...	...	...	..	..	..	..	1.2	8.8	9.0	102.6
23	...	...	...	..	..	..	..	4.2	9.2	8.2	98.0
24	1.08	2.9	10.0	93	27	29	88	4.6			

TABLE 3.—*The mean and standard error of various erythrocyte values before and after splenectomy and following infection with* B. canis

	Erythrocytes		Hemoglobin		Vol. packed erythrocytes		Mean corp. volume		Mean corp. hemoglobin		Mean corp. Hb. Conc.	
	Mean	σ m	Mean	σ m	Mean	σ m	Mean	σ m	Mean	σ m	Mean	σ m
Before splenectomy	5.86	0.22	12.3	0.50	45.7	1.50	78	2.7	20	1.2	27	1.2
After splenectomy	6.03	0.19	12.6	0.57	46.0	1.22	77	2.6	21	1.3	27	1.7
$\frac{\text{Difference}}{\sigma \text{ difference}}$	0.58 σ		0.41 σ		0.15 σ		0.27 σ		0.58 σ		0.0 σ	
Before infection	6.03	0.19	12.6	0.57	46.0	1.22	77	2.6	21	1.3	27	1.7
After infection (death)	2.00	0.29	4.1	0.61	15.6	2.16	81	4.8	22	1.5	26	0.6
$\frac{\text{Difference}}{\sigma \text{ difference}}$	11.8 σ		10.2 σ		12.2 σ		1.1 σ		0.5 σ		0.5 σ	

hypothesis concerning the susceptibility of the human reticulocyte to the malarial parasite, an attempt was made to determine whether this happened in piroplasmosis, i.e., did the parasites show a greater affinity for the immature erythrocytes? This aspect will be considered later.

After inoculation, the parasites could be observed in the blood smear usually within one or two days. The presence or absence of a spleen, as well as the age of the animal, seemed to be associated with the number of parasitized erythrocytes in the peripheral circulation (Table 4). It

TABLE 4.—*The percentage of infected erythrocytes in the peripherial circulation of dogs infected with* B. canis

Days after infection	Percentage of parasitized erythrocytes											
	Spleen absent								Spleen present			
	Adults							Immature	Adults		Immature	
Dog	216	233	251	252	273	280	305	224	254	268	246	223
1 ..	0	0	0	0	0	0	0	..	0	0	0	0
2 ..	0.2	0	0.4	2.4	2.8	2.6	11.2	..	0.2	0.4	1.6	0.2
3 ..	2.4	0	4.2	23.6	6.2	1.6	20.8	..	1.0	0.2	0.4	2.2
4 ..	0.4	0.6	0.5	0.6	10.0	0	0.4	..	0	0.4	0.2	0.6
5 ..	0.2	1.4	0.6	1.2	1.8	0	0.6	..	0	0.2	0	0.4
6 ..	0.6	0.2	0.8	0.6	2.8	0.2	3.6	1.0	0	0	0	0.2
7 ..	0.6	13.8	0.2	0.2	1.2	0	0	1.2	0	0	0	0.2
8 ..	0.2	1.0	0	0.6	7.8	...	...	0.2	0	0	0.2	0.4
9 ..	0.6	0.2	0.4	0.2	1.6	0	0	1.0	0	0.2	1.2	0.4
10 ..	0	0	0.4	0.8	...	0	0	1.0	0	0	1.8	0.2
11 ..	0.6	0	0.4	0	...	0	0	2.1	0	0	2.4	0
12 ..	0.2	0	0	...	...	0	0	0.6	0	0	17.6	7.8
13 ..	1.2	0	0	0	...	0	...	1.0	0	0	16.2	1.4
14 ..	1.4	0.2	0	0.2	...	7.6	0.8	0.2	0.2	0	...	2.4
15 ..	5.4	0	0	0	...	21.8	...	0.6	0	0	...	3.6
16 ..	1.6	0	0	0	...	...	...	1.0	0.2	0	...	..
17 ..	4.8	1.2	2.0	0	...	...	...	0.8	0	1.2	...	..
18 ..	7.0	5.8	1.0	0	...	...	...	1.2	0.2	0	...	..
19 ..	2.2	5.0	17.4	6.6	...	...	...	2.0	0	0	...	..
20 ..	6.0	5.8	...	1.2	...	...	...	0.6	4.8	..	...	..
21 ..	6.0	...	...	23.2	...	...	...	3.8	0.2	..	...	..
22 ..	1.8	...	...	17.8	...	...	...	4.2	0	..	...	..
23 ..	1.0	...	...	17.4	...	...	...	1.8	0	..	...	..
24 ..	2.2	...	...	...	...	...	...	0.8	0	..	...	..
25 ..	1.6	...	...	...	...	...	...	..	0	..	...	..
26 ..	1.0	...	...	...	...	...	...	..	0	..	...	..
27 ..	1.8	...	...	...	...	...	...	..	0	..	...	..

was observed that the splenectomized adults had a greater percentage of infected erythrocytes present than adults of a similar age with a spleen (Table 4).

The maximum percentage of infected cells found in the splenectomized animals was 23.6 per cent as compared to 4.8 per cent in the adult animals having a spleen (Table 4); in fact, each adult canine without a spleen showed a greater number of parasites than the highest recorded for the two adult, non-splenectomized animals.

It may be pointed out also that in general the parasitized erythrocytes, observed in the adult splenectomized animals, followed a definite pattern. The number gradually increased until it reached a maximum about the third day after inoculation, then dropped to a fraction of a per cent and continued thus until several days before death when it again increased

(Table 4). On the other hand, the adult animals having a spleen did not show such a distinct trend; the percentage of infected cells being very low or zero (D254, D268, Table 4). The young animals (D246, D223) having a spleen show, however, the same pattern as the splenectomized adults but the increases were not quite as marked.

There was no evidence of periodicity on a twenty-four hour basis or longer period.

DISCUSSION

Since a number of the animals in the present study had been subjected to splenectomy, the effect of the removal of this organ upon hematopoiesis must necessarily be considered. From evidence accumulated on thirty-two dogs, Krumbhaar (1932) found that, in the normal animal, splenectomy caused a true, mild, temporary anemia that varied not only in different individual dogs, but in time of appearance, degree and duration. In his series Krumbhaar did not report any deaths from anemia.

In the present series the removal of the spleen seemed to have little effect upon the erythrocyte values of the uninfected dogs. The mean and standard deviation for these values, on all dogs prior to and following spleen removal, is given in Table 3. When tested by the usual statistical procedure, previously cited, the means were not significantly different. To be significant the value of sigma must be greater than three; however, in these studies the value of sigma in all instances was less than one (Table 3, line 3); a variation no greater than one would expect by random sampling.

To test further the effect of splenectomy, this organ was removed from an adult dog whose blood had been studied previously. The blood picture was followed for thirty-five days after the operation. Over this period the change in the total red cell count was about half a million and for the hemoglobin was less than two grams. Before the 35th day these components had returned to the previous normal level. It appeared obvious, therefore, that the marked anemia observed in the piroplasmosis infections was brought about by the presence of the parasite and not by the removal of the spleen.

As pointed out previously, no definite trend was shown by the corpuscular constants although the anemia increased constantly. Since there was no change in the corpuscular constants the anemia may be classified as "normocytic." In his classification of the anemias, Wintrobe (1934) gave as one of the things responsible for a normocytic anemia, acute destruction of the blood, citing hemolytic anemia caused by malaria as an example. On the basis of the hematological findings the anemia could be brought about by a similar mechanism, i.e., the destruction of the erythrocytes by the protozoan parasite. Whether or not the anemia, per se, was responsible for the death of the animal cannot be stated cate-

gorically at this time without complete pathological studies. Anemias of such magnitudes, however, have been responsible for the deaths of canines in uncomplicated cases of hookworm disease and the blood loss alone (Landsberg, 1937).

In the present studies we have been unable to demonstrate the affinity of the parasite for the immature erythrocyte. The incidence of parasitized reticulocytes was low, in fact, in eight of the experimental animals no such cells were found at all during the entire period of infection (a total of 130 reticulocyte counts). In the remaining four animals, parasitized reticulocytes were observed in sixteen instances (a total of 78 reticulocyte counts), which involved a total of 79 reticulocytes.

The data were examined further and the correlation between the percentage of reticulocytes and parasites determined. It was found that the correlation coefficient was 0.26 which did not indicate a high degree of correlation. As a different approach to this problem the cell diameters were measured. It was observed that the diameter of the non-parasitized, adult canine erythrocytes averaged 6.80 microns, a figure agreeing exactly with that given by Jordan (1938). The non-parasitized reticulocytes had an average diameter of 7.25 microns (Table 5). When

TABLE 5.—*The mean diameter and standard deviation of adult, normal, reticulated and parasitized erythrocytes*

	Number measured	Mean diameter (microns)	σ indiv. (microns)	σ mean (microns)	$\frac{\text{Diff.}}{\sigma \text{ diff.}}$
Adult erythrocytes (1)	1,192	6.80	1.09	.031	.....
Reticulocytes (2)	1,116	7.25	0.98	.029	(1) (2) = 10.7 σ
Erythrocytes with one parasite (3)	1,244	7.03	1.14	.032	(2) (3) = 5.1 σ

tested statistically (difference, divided by σ of the difference) the reticulocyte was significantly larger than the adult erythrocyte (Table 5). The erythrocytes containing one parasite, although significantly larger than the uninfected cell (Table 5) were significantly smaller than the reticulocyte. Although such criteria are not infallible, since theoretically the entrance of the parasite into the reticulocyte could bring about a reduction in the size of the reticulocyte (or a loss of granules) the size differentiation supported the view that the young red cell infection was probably a fortuitous circumstance.

The idea of fortuitous parasitism of the immature erythrocyte is not shared by Eaton (1934a), however, who states, "The piroplasms showed an especial predilection for reticulocytes, comparable to that which I have recently shown to characterize the human malaria parasites. . . ." On the other hand, Simons (1939) found that the babesia did not invade the reticulocytes even though in some animals 23 per cent reticulocytes were present; a percentage comparable with those of the present study.

The present observations agree essentially with those published by Saunders (1937) who reported a marked anemia, an enlarged and somewhat softened spleen, an absence of parasites in the peripheral circulation until several days prior to death when the number of parasitized erythrocytes increased greatly and, finally, that splenectomy brought about increased numbers of parasites in the peripheral circulation.

SUMMARY

The erythrocyte picture of dogs infected with *Babesia canis* was studied in detail. It was observed that all animals died as a result of infection with this parasite and a marked anemia was present in all, irrespective of the presence or absence of the spleen. The anemia was "normocytic" in nature. The parasites were present in the mature erythrocytes and did not appear to have a predilection for the reticulocytes. In the splenectomized mature dogs and the immature animals with the spleen intact the greatest number of parasites was observed just several days prior to death. The temperature remained above normal the entire period of infection dropping only just before death.

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TRICHODINA URECHI N. SP., AN ENTOZOIC CILIATE FROM THE ECHIUROID WORM, *URECHIS CAUPO*

GLENN A. NOBLE

Trichodina urechi n. sp. is a peritrichous ciliate which lives in the mid-gut of the echiuroid worm *Urechis caupo* Fisher and MacGinitie, 1928. Most of the worms used were taken from Drake's Bay (longitude 122° 57′, latitude 38° 4′), California, but some were secured from Elkhorn Slough (longitude 121° 46′, latitude 36° 49′) and Morrow Bay (longitude 120° 52′, latitude 35° 20′). The *Trichodina* are usually limited to the posterior third of the mid-gut of the host. This portion of the intestine differs slightly in color from the rest of the gut, and its contents are more viscous. It frequently contains numerous fecal pellets over which *Trichodina* actively move. The greatly distensible hind-gut, often filled with sea water, does not contain any protozoa, and the portion of the mid-gut anterior to the habitat of *Trichodina* contains a greenish or brown fluid which is apparently unsuited to the ciliate. It may be that the food material in the mid-gut has not yet reached a condition in which it is useful to the protozoa. It is also possible that this habitat is detrimental to the reproductive processes of the ciliate. If experimentally placed in this fluid, however, the protozoa live almost as long as when placed in fluid from their normal habitat.

The method of infection of young worms by the commensal has not been determined. The usual method by means of a cyst suggests itself, but as yet encystment among *Trichodina* is unknown. No reproduction of the ciliate, taken from the host under laboratory conditions, could be detected. This may be correlated with the fact that in the laboratory apparently no ciliates emerge from the host. Discharges from the gut of *Urechis* were examined, and the water in which several hosts were living was repeatedly centrifuged and scanned without positive results. Since the protozoa live for but a short time in sea water, however, and may be quite sensitive to centrifuging, they may have disintegrated before their observation was possible. Small very young *Urechis* were found to be without *Trichodina,* which fact indicates that infection is associated with a certain size or stage in the life cycle of the host.

TECHNIQUE

For intra-vitam staining, it was found that neutral red gave the best results, although methyl green, Janus green, and Bismarck brown were satisfactory. Each of these stains was used in a 1/10,000 dilution in Ringer's fluid. Food vacuoles were the first to take the stain; then the

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macronucleus and denticle ring stained deeply. A Taylor microdissection apparatus was used on both living and stained forms. The latter material was removed from slides and placed in dilute balsam.

Permanent whole mounts were made by spreading some of the gut material thinly on a slide and fixing it in Schaudinn's solution. *Trichodina* were also obtained in larger numbers through centrifuging some of the gut contents. Heidenhain's iron-haematoxylin was found to be the most satisfactory stain. Good results were obtained with Delafield's haematoxylin, with Harris' haematoxylin, with Mallory's triple connective stain, with thionin, and with borax carmine. Fixation in either Bouin's or Zenker's fluids was equally satisfactory. Silver and gold stains, modified for marine organisms, however, were not satisfactory. Feulgen's stain aided in the observation of the nuclei. Serial sections were made of the intestine of *Urechis* using the paraffin method.

MORPHOLOGY

Trichodina urechi measures about 40 (25–50) microns in length, while its width, as seen in an oral or aboral view, is about 58 (48–75) microns. The general shape of the body may be likened to a turban (Plate II, Figs. 2 and 4). There are, however, many variations from this shape, depending on the activity of the animal. Because of the motion of the ciliate and its peculiar structure, the terms anterior and posterior may be somewhat confusing. For that reason the end which is usually called anterior is here termed oral while the attaching, or posterior, end is designated as aboral. In *T. urechi* the oral end is usually rounded or dome-shaped with the opening to the cytopharynx located laterally at the base of the dome.

Cilia within the cytopharynx are continuous with the ring of cilia which encircles the base of the oral dome. This ring of cilia forms a left-handed spiral* into the cytopharynx as viewed from the oral surface. This spiral arises from the floor of a deep narrow groove (Plate II, Fig. 4). The ring makes one complete turn of the body before descending spirally into the cytopharynx.

On the aboral surface of *T. urechi* is located a cup whose walls are parallel to the long axis of the body. *Trichodina* are frequently studied after the smear has become dried on the slide. In this condition the aboral cup has become flattened out so that the whole posterior complex appears more like a disc than a cup. The advantages of a deep cup become apparent when one observes the remarkable adhesive power of this organ.

Within the walls and the floor of the aboral cup are located numerous rays or rods. In the flattened, dried condition these rods appear as rings

* The outside portion is here considered the beginning of the spiral.

of radiating lines concentric with the denticle ring (Plate I, Fig. 1 *supporting rod*). Thus the system of fibers within the wall of the cup is frequently called a "striated ring" (Mueller). This description gives the impression that it is a ring in the same sense that the denticles form a ring, which is strictly not true. The relationship of the fibers to the denticle ring as it appears in *T. urechi* is described below.

The *integument* is a thin, tough, transparent pellicle which rarely shows minute striations. When pushed by a microneedle, it withstands considerable pressure. Disintegration follows rapidly as soon as the pellicle has been broken.

Cilia occur as follows: (1) An oral ring, the *oral ciliary ring,* which spirals into the cytopharynx (Plate I, Fig. 2); (2) Two rows within the cytopharynx; (3) Two concentric rings, one large, the *aboral ciliary ring I,* and one small, the *aboral ciliary ring II,* around the edge of the posterior cup (Plate I, Fig. 1, and Plate II, Fig. 4); (4) A semicircle of small cilia arising from the floor of the aboral cup along the edge opposite the cytopharynx as seen in an aboral view (Plate I, Fig. 1).

The cilia of the *oral ciliary ring,* on the oral end of the body, arise from the floor of a narrow, deep groove which surrounds the base of the anterior dome. These cilia are difficult to see in the living animal because they are very delicate and usually cease to move soon after the ciliate is removed from the host. When fully expanded, they project orally beyond the tip of the dome. The basal granules of these and of other cilia can rarely be distinguished because they lie in granular bands, which are stained deeply and follow the course of the cilia. Mueller (1938) has made the suggestion that these pigmented bands are in reality myonemes. McDonald (1922), working with *Balantidium coli,* suggests a conducting function for similar granular bands of protoplasm.

The cilia on the oral surface continue into the cytopharynx and there divide into two rows (Plate I, Fig. 2). The first two form the main body of the cytostomal cilia. These cilia are arranged in the form of membranelles. They lie on the inner wall of the cytopharynx and are separated by a slight irregularity of that wall. The third division is a tuft of cilia arising at the end of the cytopharynx. These cilia form a long, whip-like bundle which is apparently active only when food vacuoles are being formed. The second row of cilia in the gullet projects at right angles to the first row. It arises from the outer margin of the cytopharynx, i.e., the margin forming the greater curvature. Except for the inner tuft, these cilia beat continuously and serve to draw food particles into the body.

The aboral rings of cilia are the main locomotor organelles. *T. urechi* is similar to *T. urinicola* Fulton (1923) in that this ciliary crown is made up of true cilia. They differ, however, in that the former has two rings,

an outer one composed of short thick cilia, and an inner one whose cilia are long and fine. A mass of granular cytoplasm accompanies the basal granules of these cilia as is the case with those of the oral surface. This mass stains readily and is located nearer to the basal granules of the inner circle of cilia than to the outer. The cilia of the outer ring are spaced comparatively wide apart, while those of the inner ring are so close together it is nearly impossible to differentiate them. Under normal conditions the cilia beat with a wave-like motion, although those of the outer ring do not necessarily beat with the same rhythm as those of the inner ring.

The semicircle of cilia on the floor of the pit can be seen in the living animal only when the cilia are moving. Even in stained and sectioned specimens these organelles easily go unnoticed. Such a semicircle of cilia is not unknown in other *Trichodina.* In describing *T. myakkae,* Mueller (1937, p. 180) says, "The adoral spiral of cilia could not be observed very successfully in whole mounts, but appears in transverse sections on one side of the disc only. It evidently does not form a complete circuit of the anterior face, traversing only about half of the circumference." The function of this semicircle in *T. urechi* is difficult to ascertain. It may be that by beating within the cup a current is created, which assists the animal in detaching itself from another *Trichodina,* from fecal matter, or from the intestinal wall.

The *cytoplasm* is usually very granular, a condition which varies, however, with different stages of the life cycle. Just before division the cytoplasm becomes lighter in appearance and more homogeneous than usual. This change is characteristic of ciliates in general, and in *Colpoda duodenaria,* according to Taylor and Garnjobst (1939), it marks the reorganization process which includes not only the cilia but the entire "silverline system." Otherwise the granules are of different sizes, mostly small.

Food vacuoles are usually numerous throughout the body. The food vacuoles contain granules of various sizes consisting of bacteria, diatoms, and fecal matter from the host. Generally these vacuoles are located more toward the periphery of the cell than near its center, and usually a large mass of them lies in the dome-like extension of the oral end, although this end sometimes appears free of all vacuoles. The number of food vacuoles within an individual may usually be directly correlated with the number of fecal pellets within the host.

In addition to these structures there is a conspicuous, large, clear vacuole situated near the end of the cytopharynx (Plate II, Fig. 4). It measures about 14 microns in diameter and appears spherical. No contraction of the vacuole could be observed and no outlet could be found.

The *macronucleus* has, roughly, the appearance of the letter H when

seen from the oral or aboral side (Plate I, Fig. 1). Each lateral piece takes the form of a shallow crescent with slightly bulbed ends. An enlarged area also occurs where the cross piece of the H joins the lateral segments. Thus from a lateral view (Plate II, Fig. 4) it can be seen that the middle and lateral segments do not lie in the same plane. The distance across the macronucleus averages 46 (40–55) microns. This nucleus consists, during the inter-fission period, of numerous small bodies imbedded in a homogeneous-appearing matrix. With iron haematoxylin these bodies appear as black dots. When destaining the macronucleus of *T. urechi,* it was found that the middle piece became lighter in color than the rest. This was the case with both iron haematoxylin and Feulgen's stain. This difference in affinity for dyes indicates a difference in structure and in turn suggests a difference in function among various portions of the macronucleus. Another evidence for such a division of labor is the fact that when the macronucleus disintegrates, it is this central part which usually disappears first.

The *micronucleus* is a small, compact, oval body located near the end of either of the lateral pieces of the macronucleus, although it is usually found, in an aboral view, opposite the middle of the outer margin of the cytopharynx (Plate I, Fig. 1). It never lies within a depression of the larger nucleus as is the case with several other species of *Trichodina* (e.g. *T. patellae* Cuenot). This structure in *T. urechi* stains more deeply than the macronucleus. The small dots characteristic of the latter structure cannot be seen in the micronucleus. Chromosomes are visible only during division.

The *denticle ring* stands out distinctly as a deeply colored ring, half the circumference of the body. It lies beneath the floor of the aboral pit, at the base of its walls, and in living animals it appears highly refractile. The ring is composed of 18–25 pieces or denticles so arranged as to give it the twisted appearance of a rope. Each denticle possesses a narrow blade-like process which projects aborally. These processes correspond to the hooklets or *uncini* (Fantham, 1924) of other species of *Trichodina.* Since they are very thin, they usually appear lighter in color than the rest of the ring, and their position renders them unobservable from an oral or aboral view. They can be seen in longtitudinal sections although they are frequently bent or destroyed. Diller (1928), Fulton (1923), and Mueller (1938) describe each denticle as possessing a hook and a ray, the latter pointing centrally. In *T. urechi* the centrally pointing rays appear to be continuations of the supporting rods, described below. Each denticle is applied so closely to the ones adjacent to it that the division lines are frequently obscured. When teased apart with the microdissection needles, however, the ring reveals its true composition of distinct units. Little is known of the chemical composition of this denticle ring or corona.

There are two other ring-shaped structures which surround the walls of the cup near the rim. As with the denticle ring, the function of each of these other two rings is assumed to be, in part, supporting. They are called the *basal ring* and the *aboral ring* (Plate II, Fig. 4). The basal ring lies at the base of a thin velum and near the origin of the main circlet of cilia. This ring stains so deeply that its finer structure could not be ascertained. The aboral ring is wider, much lighter in staining properties and lies orally with respect to the basal ring. In individuals pulled apart with the micromanipulator both of these rings, or pieces of them tend to maintain their integrity. A possible clue to the structure of the basal ring is given by a study of a somewhat similar band of material which accompanies the oral ciliary spiral. In this case there is a central fiber (or fibers) densely surrounded by granules. Here the fiber is probably neuromotor in function. In dividing animals it can be seen that the aboral ring is composed of enlargements on each of the supporting rods noted below.

Another set of structures whose size and location suggest a supporting function and which in addition probably play a role in the neuromotor system is the *supporting rods,* which originate in the center of the floor of the aboral cup (Plate I, Fig. 1, and Plate II, Figs. 1 and 4). These rods or fibers radiate to the base of the pit wall and bend sharply to line the wall. There is a swelling or granule in a similar position on each rod. These granules are adjacent to one another and so form a ring, the aboral ring noted above. Each rod finally ends in a minute granule touching the basal ring. There are 150 to 200 of these rods, approximately eight to every segment of the denticle ring, which lies medially to the rods and is in contact with them. By means of the microneedles portions of the denticle ring may be pulled out with rods attached. The function of these fibers is suggested by the term "ribs" as used by Fulton. Certainly whatever other function they may have, such comparatively rigid structures lying in the cup wall must necessarily serve as a support in some degree. In certain ciliates (*T. renicola* Mueller, 1932, and *Vauchomia nephritica* Mueller, 1938) there is a series of "anterior rays" lateral to the supporting rods ("striated ring"). Mueller calls these "soft rays" and says of them (1938, p. 253): ". . . I am unable as yet to arrive at definite conclusions, but it now appears most likely that these soft rays are a system of myonemes connected with the posterior girdle of cilia." If these soft rays are myonemes, it is possible that the supporting rods of *T. urechi* are also myonemes. This interpretation conforms to the relationship these fibers have to the neuromotor system.

A thin velum arises from the basal ring. It can be seen only in sectioned individuals where it appears as a continuation of the wall of the aboral cup. The function of the velum has not been ascertained. Its

location suggests that possibly it plays a part in the attachment of the animal to various objects.

The *cytopharynx* spirals aborally from the base of the anterior dome until it reaches the middle of the body, then curves orally for a short distance. When seen from an oral or aboral view, the outer margin of the cytopharynx is an even curve while the inner margin possesses three secondary curves corresponding to the three groups of cilia (Plate I, Fig. 2). The innermost portion of the cytopharynx remains constricted when food vacuoles are not forming. *Trichodina* are frequently fixed while their bodies are not in the usual position but shifted to one side, usually to the side upon which the mouth opens. This fact accounts for the appearance of the cytopharynx at the outermost edge of the body when one is studying fixed and stained material (Plate I, Fig. 1). In several *Trichodina* such as *T. pediculus* (Mueller, 1786) Ehrb., 1838 the cytostome opens into a vestibule and this in turn into the gullet or cytopharynx. In *T. urechi* there is no such anterior chamber or vestibule. The diameter of the cytopharynx, however, is not constant. With alterations of the shape of the entire body there occur alterations of the shape of the gullet, so that at times it is wide with ample room for activity of cilia within it, and at other times it appears to be practically closed.

NEUROMOTOR SYSTEM

In *Trichodina urechi* the various fibrils, granules, and possibly pigmented bands constitute the neuromotor system. Almost all of these structures are connected directly or indirectly with a centrally placed granule, larger than the others, which corresponds to the neuromotor motorium as described in other ciliates (Sharp, Yocom, Taylor). This motorium is located near the end of the cytopharynx where the last tuft of cilia arises (Plate II, Fig. 5). Since most animals are fixed with the inner chamber of the cytopharynx closed, the motorium usually appears as a darkly-stained, small mass lying over the proximal end of the gullet. From the motorium project two horns, which continue as two fibers that connect the basal granules of the two cytopharyngeal ciliary rows. These fibers join near the cytostome and, as one strand, connect the basal granules of the oral spiral of cilia. A band of granules obscures this strand.

Many wavy fibers which apparently play the role of myonemes connect the oral spiral to the denticle ring. They are not located at the periphery of the cell. The varied activity of the body suggests the presence of such contractile threads or myonemes. The location of these threads in *T. urechi* indicates that they are also conductive in function. They form the main connection between the locomotor complex of the aboral end of the animal and that of the oral surface. This dual role of myonemes is suggested by Calkins (1933) as follows: "It is conceivable,

moreover, that myonemes in a generalized condition may be both coordinating and contractile in function." These fibers are somewhat similar to an internal supporting basket described by Peschkowsky (1923) for *Trichodina*. Stevens (1904) has described for the peritrich *Lichnophora* a comparable system of fibrils which connect the oral spiral with the posterior disc. Probably stimuli pass along the myonemes in *T. urechi* to the denticle ring and from there to the supporting rods described above. Here again a dual function is assumed for a system of fibers. The connection of the supporting rods to the basal ring, which in turn is connected by minute fibrils to the basal granules of the large aboral ciliary ring, recommends the assumption that these supporting rods transmit stimuli. It is possible that some of these fibers are purely supportive in function while others conduct impulses. This interpretation is strengthened by the observation that in some *Trichodina* alternate fibers appear to be slightly narrower than the others with the suggestion of a swelling on each one. No fiber can be seen connecting the basal granules of the secondary or outer ciliary ring with those of the primary aboral ring. A fiber does, however, connect the basal granules of the former ring with each other.

As yet no one can give an unqualified statement as to the function of this system of fibers. In speaking of the work of Sharp (1914), on *Diplodinium ecaudatum,* Calkins states: "The delicacy of structure and the position of this amazingly complex aggregate are sufficient evidence to disprove any hypothesis of a supporting function." Yet when examining crushed or disintegrating *T. urechi* or tearing them apart with the microdissection needle, one can see that many of the fibers remain intact, a fact which demonstrates a compact and more durable composition than the rest of the cytoplasm. Some degree of support is probably furnished to the body by the mere presence of fibers of sufficient density to withstand pushing about within the cytoplasm. That they also play a part in the motor activities of the body suggests itself. In particular, the fibers which connect the oral cilia with the denticle ring seem to be contractile threads because of their wavy appearance. It may be that the basal ring is also a myoneme. Mueller (1938) describes a similar dense band of protoplasm in *Vauchomia nephritica* as "probably . . . also a myoneme, and its action opposes that of the radial myonemes" (p. 255).

Thus, in *T. urechi* there is a neuromotor system which compares favorably with that described for other ciliates. Except for the outer aboral ciliary ring, all the cilia are connected with each other and unified by a system of fibrils which apparently originates in a centrally placed body or motorium. No peripheral "silverline" fibrils could be demonstrated.

BEHAVIOR

There are a few peculiarities in behavior which distinguish *Trichodina urechi* from most other ciliates. Under certain conditions, whose nature has not been ascertained, the majority of animals which can be seen within the field of the microscope are attached to each other by their aboral cups. Large masses or clumps of ciliates are formed, or long chains may appear. At any time various members of these aggregates may detach themselves and swim away to join some other group. Frequently two *Trichodina* will attach themselves to a third one and then will attempt to swim in opposite directions. When this happens, the body of the middle member of the trio is drawn into the sucking cups of the other two (Plate II, Fig. 6).

A periodic contraction of the body frequently occurs about once a minute, although sometimes more often. While an animal is attached to the coverslip, it slowly expands in a lateral direction. When it reaches a certain size, it will suddenly contract with a marked shifting about of the cytoplasmic inclusions.

Under favorable conditions the formation of a food vacuole can be observed. The tuft of long cilia at the proximal end of the cytopharynx lies within a portion of the gullet which is closed except during active feeding. At such times the proximal ciliary tuft beats with a wave-like, spiral motion within an expanded chamber. Food particles pass slowly through this expanded chamber and form a more or less spherical mass. When the mass reaches the end of the gullet, the ciliary tuft beats with diminished vigor, and, as the mass enters the cytoplasm, the expanded chamber contracts and appears to withdraw from the food. This departure leaves the mass within the cytoplasm surrounded by a vacuole wall. The open end of the gullet collapses, thus disappearing from view along with the ciliary tuft, which ceases beating. In the meantime, the rest of the cytopharyngeal cilia continue their rapid motion. After a few seconds the ciliary tuft resumes its activity, and the process is repeated.

REPRODUCTION

Conjugation has not been observed among *Trichodina urechi*. The only type of reproduction that has been seen is binary fission without the formation of a cyst. In general, this division is not unlike the process which occurs in other ciliates. It was found, however, only at the seaside immediately after the worms were taken from the sand.

The first indication that division is taking place in *Trichodina urechi* is an enlargement of the ciliate with an accompanying increase in homogeneity of the cytoplasm. This cytoplasmic change before division is characteristic of ciliates in general. With *T. urechi* the entire process of division is accomplished while the animal is actively swimming around.

This activity indicates that the redifferentiation of organelles takes place gradually, new structures appearing while the old are still functioning. The division process can conveniently be followed by tracing the fate of each organelle individually.

Macronucleus. After enlargement of the cell, the macronucleus begins to change from an H-shaped structure to a roughly rod-shaped mass at the center of the animal. The lateral portions of the macronucleus are the first to change. There occurs a gradual clumping of the entire nucleus to form an amorphous body (Plate III, Figs. 2–6). This mass becomes elliptical in outline and, when the cell divides, constricts to form two equal portions. It was noted above that the macronucleus consists of a homogeneous matrix in which are imbedded numerous deeply staining granules. At the onset of division these granules vary in size in different individuals but soon disappear from view, the entire macronucleus appearing solid black or grey with iron haematoxylin. This fact indicates that a reorganization process occurs in the macronucleus. The significance of the granules is doubtful, although their staining properties suggest a chromatin make-up.

Micronucleus. Soon after the macronucleus begins to alter in shape, the micronucleus enlarges and appears lightly stained compared to its interdivision period. At this time it often appears oval with a truncated end. As minute fibers appear within the micronucleus, the flattened end fills out and the nucleus forms a structure elliptical in outline with pointed ends. The fibers become increasingly apparent while chromosomes can be distinguished (Plate III, Fig. 3). These chromosomes are short narrow threads clustered so closely together they cannot be counted. A definite spindle forms, but centrioles are not visible. The chromosomes divide, and the two portions migrate to opposite sides of the cell. The deeply staining material is packed closely together, leaving a clear space which appears like a small cap on each nucleus (Plate III, Fig. 4). Connecting the two parts of the dividing micronucleus is a definite fiber, the centrodesmose (Plate III, Fig. 4). This fiber usually appears to lie within a channel formed by the separating micronucleus. As soon as the centrodesmose disappears, each new micronucleus takes a solid black color in iron haematoxylin until time for another division.

Denticle Ring. The fate of this structure has been followed by Fulton (1923) and Diller (1928). According to these authors the old denticle ring is gradually absorbed while a new one, with twice as many denticles appears as a concentric ring outside of the old one. When the animal constricts, each ring forms a figure eight, and soon after division the old inner ring, with half the normal number of denticles, disappears.

In *T. urechi* this process is fundamentally the same, but it differs in point of time. Before and during actual cleavage there is no indication

of a new denticle ring (Plate III, Figs. 1–6). If, however, one of the daughter cells is followed after division has occurred, a new denticle ring can be observed to appear outside of the old one. At first the new one is unsegmented, in this respect resembling Diller's *Trichodina* from tadpoles. The old denticle ring, possessing half the number of denticles of the new one, gradually disappears while the latter, possessing the normal number of denticles, takes its place (Plate IV, Figs. 1 and 2). Thus it is important in a study of the division process of any protozoan to look for evidences of continued reorganization in daughter cells which have completely separated.

Supporting Rods. Redifferentiation of the supporting rods is a process originating on the floor of the aboral cup and continuing toward the lip of this structure (Plate III, Figs. 3 and 4). As the new rods develop, the old are absorbed. This replacement occurs during the earlier stages of the division process.

Supporting Rings. Closely associated with the supporting rods are the basal and aboral rings. At the onset of division the basal ring is completely absorbed. The new one appears after separation of the daughter cells. The pigmented band at the base of the ring of large cilia becomes increasingly visible. Just outside of it can be seen the basal granules of the outer ring of cilia (Plate III, Figs. 1–6). The aboral ring becomes broken into discrete granules and proves to be composed of a single enlargement on each supporting rod. These granules appear somewhat similar to the "peripheral thickenings . . . on alternate radiations" described by Diller. Minute granules on the distal ends of the newly developing rods apparently unite to become the aboral ring of the new individual.

Cilia. Evidence for the redifferentiation of the aboral cilia is meager. During the division process the basal granules become more distinct than normal. Whether they participate in the production of the new is open to conjecture.

On the oral end of the body a new spiral, adopted by one of the daughter cells, is clearly a product of apparently undifferentiated cytoplasm (Plate IV, Figs. 3–6). In other works on division in *Trichodina* a consideration of the fate of the ciliary spiral and cytopharynx is either omitted completely or simply mentioned in passing. Diller states that probably part (aboral) of the old peristome differentiates into a new cytopharynx. In *T. urechi* the old cytopharynx disappears completely. There remains a deeply staining C-shaped fiber from which cilia arise (Plate IV, Fig. 4). In some individuals these cilia are so indistinguishable as to make their presence doubtful. It is likely that all of the old cilia are resorbed.

Neuromotor System. The fate of various portions of the neuromotor system has been discussed in connection with the above-mentioned structures. The motorium disappears along with the cytopharynx and is redifferentiated after the daughter cells break apart. The myonemes also are completely resorbed, being replaced during the post-separation period.

Vacuole. The old large vacuole disappears during division while just before separation a new one appears, which apparently divides to produce one for each daughter cell.

COMPARISON OF *Trichodina urechi* WITH OTHER MEMBERS OF THE GENUS

The various species of *Trichodina* have been described by many authors using techniques of different efficacy and characterized by differences in skill of observation. Many of these descriptions are far too meager to serve as a reliable basis for comparison with *T. urechi.* Others make no attempt to describe the entire animal, but discuss particular characters or certain aspects of behavior. The failure to mention the presence of a structure does not necessarily indicate its absence. For these reasons, of the numerous species of *Trichodina* to be found in the literature only the following warrant a comparison with *T. urechi: T. laborum* Chatton (1910), *T. patellae* Cuenot (1891), *T.* sp. Diller (1928), *T. pediculus* (Ehr) James-Clark (1866), and *T. urinicola* Fulton (1923). Comparisons will be made with the following diagnostic characters of *T. urechi:* (1) body turban-shaped, (2) H-shaped macronucleus, (3) micronucleus whose outline resembles that of a kernel of corn and is separate from the macronucleus, (4) a denticle ring each denticle of which possesses a blade-like process, (5) two supporting rings near the origin of the aboral cilia. None of the above authors describe a semicircle of cilia within the aboral cup.

T. laborum has a body shape similar to that of *T. urechi.* The macronucleus of the former is C-shaped and its micronucleus, which usually lies adjacent to the macronucleus, is spherical. *T. laborum* has no denticle ring, and no supporting rings were described.

T. patellae, as described originally by Cuenot (1891), has a body shape similar to that of *T. urechi.* Its macronucleus is roughly S-shaped, while its micronucleus is elliptical in outline and lies apart from the macronucleus. The denticle ring has no hooks or blades, and no supporting rings were described. The description of the same species by Caullery and Mesnil (1915) differs considerably from that of Cuenot and is much more similar to the description of *T. urechi.* According to Caullery and Mesnil, the macronucleus of *T. patellae* is H-shaped and the micronucleus is frequently imbedded in the center portion of the macronucleus.

T. sp. an unidentified species from the skin and the gills of anuran tadpoles, has a body shape somewhat like a barrel. Its macronucleus is horseshoe-shaped, and the elliptical micronucleus usually lies near one end of the macronucleus. Each denticle of the denticle ring possesses a pointed hook. No supporting rings were described. Diller's description of this species is simply an introduction to an excellent account of endomixis.

T. pediculus has a body shape similar to a spool. Its macronucleus is C-shaped. The micronucleus is roughly spherical and lies imbedded in or near the macronucleus. The denticle ring possesses narrow, pointed hooks. No supporting rings were described.

T. urinicola has an elongated body. Its macronucleus consists of two large lobes joined by a bridge at one end. The micronucleus appears as a small dot near the macronucleus. The denticle ring is small, each denticle possessing a spike-like hook. No supporting rings were described.

In view of these differences in structure between *Trichodina urechi* and other members of the genus, this ciliate is designated as a new species.

Habitat: Intestine of *Urechis caupo*.

Locality: Drake's Bay, California.

Type: San Francisco Junior College.

Paratype: Santa Barbara State College, California.

SUMMARY

Trichodina urechi n. sp., a peritrichous ciliate from the mid-gut of the annelid worm *Urechis caupo* is characterized as follows: The body is turban-shaped. Two concentric rings of cilia arise from the edge of the aboral sucking cup. There is present a thin velum median to the inner aboral ciliary ring. Two supporting rings lie in the wall of the aboral cup. A single spiral row of cilia forms the oral zone. Two rows of cilia are located in the cytopharynx, one of which is in the form of membranelles. A semicircle of cilia lies on the floor of the aboral cup. The macronucleus is H-shaped. The micronucleus is roughly ovoidal in outline and lies apart from the macronucleus. Each denticle of the denticle ring possesses a single, blade-like process. A large vacuole is present which is not contractile.

A neuromotor system connected with a motorium is present.

A study of the division process, which is characterized by dedifferentiation and subsequent redifferentiation of the various organelles, emphasizes the importance of examining post-separated forms for evidences of continued reorganization.

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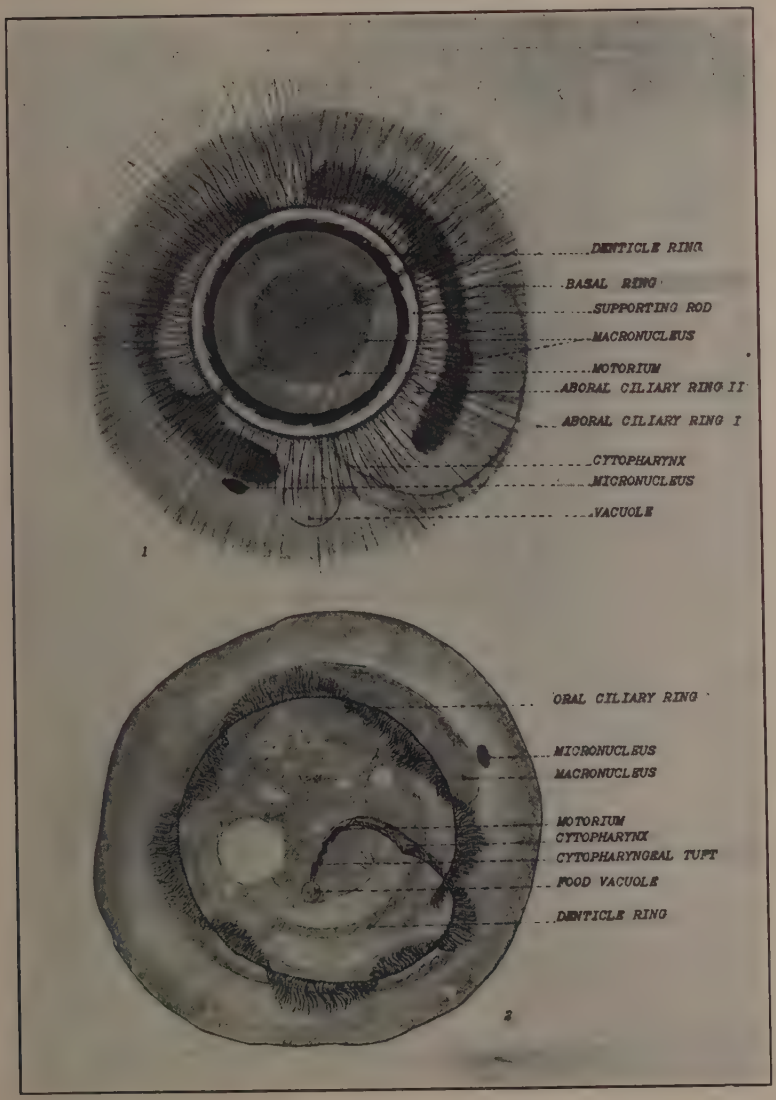

PLATE I

The outlines of both figures were made with the aid of the camera lucida.

Fig. 1. Appearance of an animal as stained with Heidenhain's haematoxylin and seen in an aboral view. ×1300.

Fig. 2. Oral view. In this drawing the oral ring and cytostomal cilia, together making the left-handed spiral, have been made considerably darker than actually appears in a prepared specimen. ×1300.

PLATE II

All but Fig. 4 are photomicrographs of *Trichodina urechi*.

FIG. 1. An animal from the aboral side revealing a portion of the supporting rods, denticle ring, and center portion of the macronucleus. ×400.

FIG. 2. Typical lateral view. ×400.

FIG. 3. An animal in the process of division. ×400.

FIG. 4. A section through the center of an individual seen in a lateral aspect. The outlines were made with the camera lucida. ×1300.

FIG. 5. A group of *Trichodina* as they appear from either an oral or aboral view. In some can be seen the neuromotor motorium. ×300.

FIG. 6. Two animals attached to a third. ×300.

FIG. 7. A low-power view of the lumen of the gut of *Urechis* showing a portion of the host tissue and a few *Trichodina* within the cavity. One is dividing. ×60.

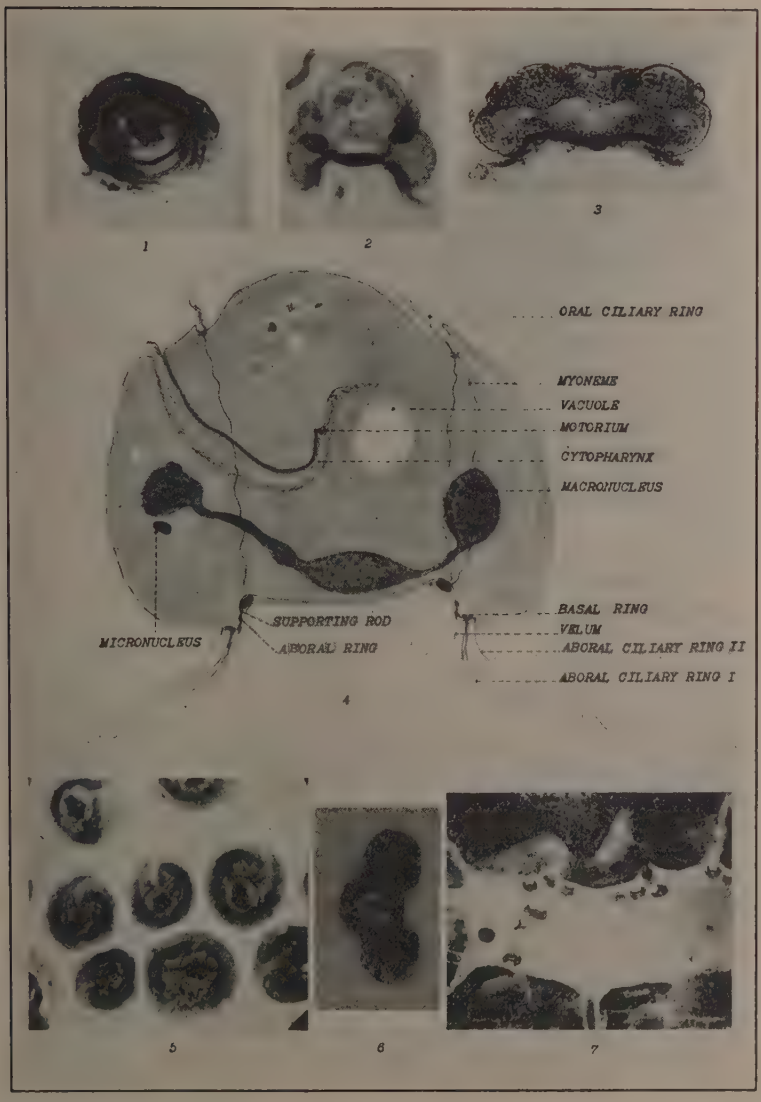
1
2
3
ORAL CILIARY RING
MYONEME
VACUOLE
MOTORIUM
CYTOPHARYNX
MACRONUCLEUS
MICRONUCLEUS
SUPPORTING ROD
ABORAL RING
BASAL RING
VELUM
ABORAL CILIART RING II
ABORAL CILIARY RING I
4
5
6
7

PLATE II

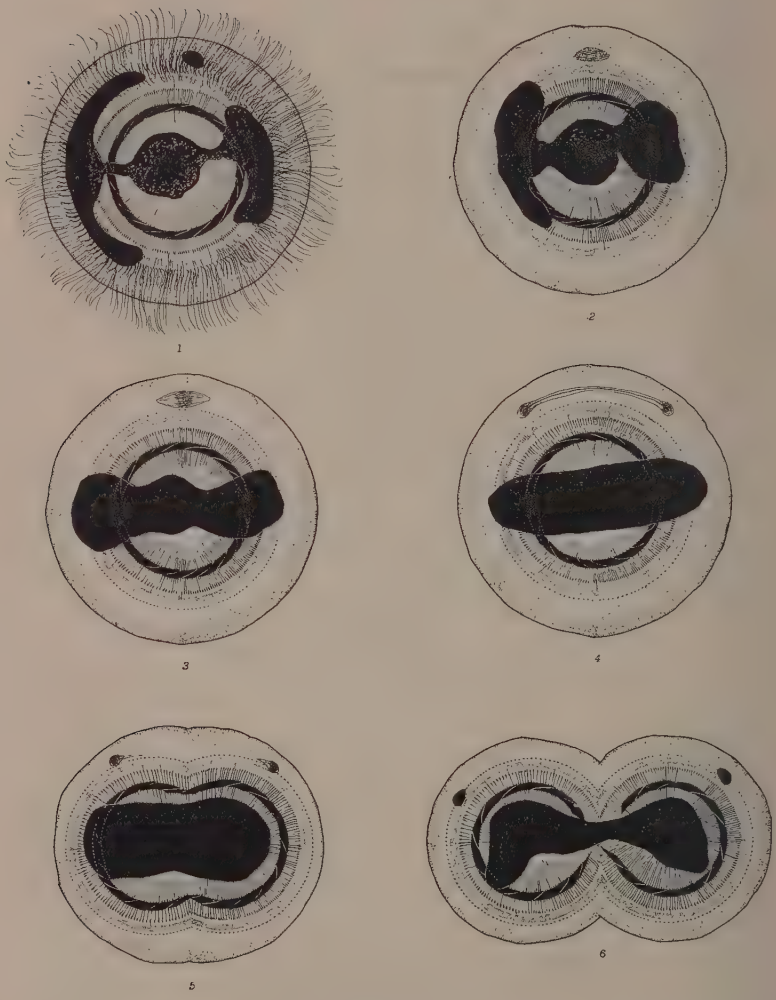

PLATE III

The figures of this plate represent the process of division up to just before separation of the daughter animals as seen from an aboral view. For clearness of representation the cilia have been omitted from all the drawings but Fig. 1. In each figure, just outside the wide pigmented band, can be seen the basal granules of the outer ring of cilia. The basal granules of the larger, inner ring are not shown for they are usually obscured by the band. $\times 600$.

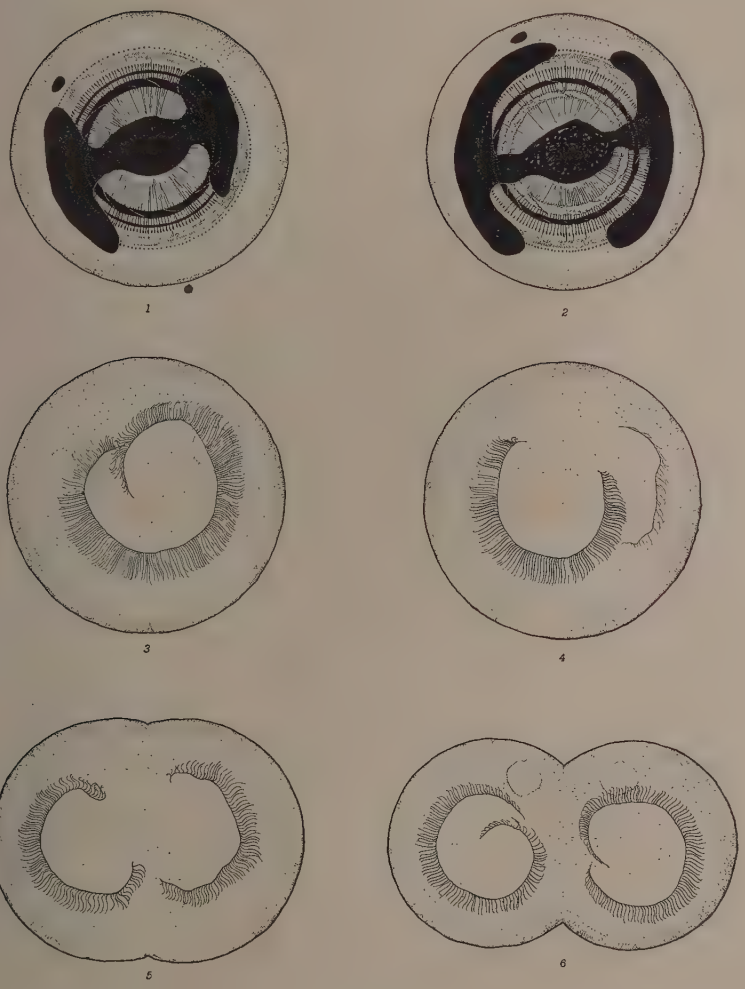

PLATE IV

FIG. 1 and FIG. 2 represent the final stages of division in that they show the resorption of the old denticle ring and appearance of the new in an individual which has completely separated from its sister cell. ×600.

FIGS. 3 to 6 illustrate the division process as seen from the oral side. The new spiral is shown to arise de novo from the cytoplasm as the old cytopharynx disappears. ×600.

THE EXCRETORY SYSTEM IN TREMATODA. I. STUDIES ON THE EXCRETORY SYSTEM IN THE TREMATODE SUBFAMILY GORGODERINAE LOOSS, 1899[1]

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Looss (1899) established the subfamily GORGODERINAE for the inclusion of the genera *Gorgodera* Looss, 1899, and *Phyllodistomum* Braun, 1899 (=*Spathidium* Looss, 1899). Later this author (1902) erected a new family, GORGODERIDAE, for the reception of the subfamily GORGODERINAE Looss, 1899, and a newly created subfamily, ANAPORRHUTINAE Looss, 1902. In the same paper Looss (1902) defined a third genus, *Gorgoderina*, as belonging to the subfamily GORGODERINAE. In this genus he included certain species formerly placed in the genus *Gorgodera*. As is pointed out in the literature, Looss (1902) mentioned for the first time the genus *Catoptroides*, a name suggested by Odhner (through personal communication). The genus *Catoptroides* was created for the reception of certain species doubtfully assigned to the genus *Phyllodistomum*.

Since the appearance of this classification for the subfamily GORGODERINAE by Looss (1902), apparently three additional genera have been described and assigned to the subfamily group. These are *Xystretum* Linton, 1910, *Macia* Travassos, 1920, and *Microlecithus* Ozaki, 1926.

Looss (1894) redescribed *Gorgodera* (=*Distomum*) *cygnoides* Zeder, 1800, and *Phyllodistomum* (=*Distomum*) *folium* v. Olfers, 1816, giving a general account of the type of excretory system each possessed. From the descriptions and illustrations it is clear that Looss was unable to make out the complete details of the excretory systems in those species. The description of the system is more complete for *Distomum cygnoides* than is the account for *D. folium*. In considering *D. cygnoides*, Looss (1894, p. 60) states: "Die Zahl der von einem Nebengefässe ausgehenden Capillarröhen ist, soweit ich sie zu zählen vermochte, nicht constant; die

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geringste Zahl war vier, die grösste acht. Bei der Kleinheit und der ein Bezug auf seine Sichtbarkeit zeimlich unbeständigen Beschaffenheit des Gegenstandes, ist ein Irrthum aber nur zu leicht möglich, und es werden mir demnach wohl hie und da Trichter entgangen sein. Nehmen wir aber für die jedem Nebengefäss zugehörigen Trichter nur die Durchschnittszahl von sechs an, dann würde sich die Gesammtzahl der Flimmertrichter für den Körper als $6 \times 12 \times 2 = 144$." In regards to the accessory collecting tubules Looss made the following statement: "Sie vertheilen sich so auf das Hauptgefässe, dass dessen vorderer Ast ihrer drei, der bedeutend längere hintere Theil sieben erhält; im ganzen sind ihrer also, mit Hinzurechnung der Enden der Hauptgefässe, zwölf vorhanden." Fig. 125, Plate VI, well illustrates Looss' convictions on the number and arrangement of the accessory tubules as well as the inconstant number of capillaries with terminal flame cells arising from each of the accessory tubules branching off from the posterior main tubule.

Nybelin (1926) first suggested that the genus *Catoptroides* should be considered synonymous with *Phyllodistomum*. He states: "Bei unserer gegenwärtigen, erweiterten Kenntnis der Phyllodistomen scheint es mir also kaum berechtigt, *Catoptroides* als selbständige, dem Genus *Phyllodistomum* ebenbürtige Gattung aufrecht zu erhalten." This view has been accepted by Lewis (1935) and Lynch (1936), both of whom considered the characters used for the separation of the genus *Catoptroides* from *Phyllodistomum* to be "too inconsequential and variable" for the establishment of the former genus.

Faust (1932, p. 466) states: "Certain genera such as *Phyllodistomum*, at present placed in the family Gorgoderidae, . . . have known excretory patterns but cannot be allocated because they stand alone in their formula groupings." This statement is interpreted to mean that the known details of the excretory system of *Phyllodistomum* would not permit its being classified as belonging to the same family as the genus *Gorgodera*. Indeed, such a classification would be impossible should the type of excretory systems described by Looss (1894) for these genera be accepted.

Sturges (1897) accepted in part the views expressed by Looss (1894) and described in some detail the type of excretory pattern to be found in *Phyllodistomum* (=*Distomum*) *patellare*. In the description and illustration of this new species we are led to the conclusion that there is a smaller number of accessory tubules originating from the anterior than from the posterior main collecting tubule, and that there is an inconstant number of capillaries branching off from the accessory tubules. Accordingly this author wrote: "The capillaries are not usually given off from short secondary or tertiary ducts as in *D. cygnoides*, but leave the primary or secondary trunks directly, so they are often of considerable length. The number of flame cells varies and is seldom the same on both sides; there are usually from twenty-five to thirty on a side."

In describing *Gorgoderina tanneri,* Olsen (1937) very briefly described the excretory system of his new species as follows: "Excretory bladder Y-shaped, arms very short, stem long and slender terminating ventral to anterior testis, viewed laterally it is sigmoid in shape with a median dorsal bend. Flame cell pattern $2[(4+4+4+4)+(4+4+4+4)]$." This author gave an excellent illustration of the excretory system of the species, from which the details are readily made out. The system in *G. tanneri* differs from the type of excretory system described for *Gorgodera cygnoides, Phyllodistomum folium,* and *P. patellare* in that there is a constant number of accessory and capillary tubules and a regular number of flame cells per group, as well as a constant number of groups on each side of the body.

Yamaguti (1934) considered the genus *Microlecithus* Ozaki, 1926, synonymous with *Phyllodistomum* Braun, 1899, since "Ozaki's description is based on a series of sections and a whole mount undoubtedly flattened under a coverslip and consequently subjected to artificial disfigurement."

Pande (1937) concluded that the genus *Gorgoderina* should be dropped in favor of the genus *Phyllodistomum.* This author states: "A comparative study of the latter genus (*Gorgoderina*) and *Phyllodistomum* has convinced me of the necessity of dropping the genus *Gorgoderina,* particularly when there are some species known belonging to the genus *Phyllodistomum* from fishes which are characterized by possessing non-spatulate body. Opinions regarding the identity of these two genera had been expressed by Osborn (1903) and Ssinitzin (1905). The former considered *Gorgoderina translucida* Stafford, 1902, and his *Phyllodistomum americanum* from the urinary bladder of the salamander, *Amblystoma maculatum,* as belonging to the same genus, while the latter compared *Gorgoderina vitellilobum* (*Distomum vitellilobum* Olsson, 1876) and *P. folium,* the type-species of *Phyllodistomum,* and decided to include *P. folium* under *Gorgoderina.* According to Cort (1912) *'G. translucida* and *P. americanum* seemed almost to constitute a transition between these two genera.' "

Recently (Summer, 1939) the writers were fortunate in securing a considerable number of favorable specimens of the species *Gorgodera amplicava* Looss, 1899, *Phyllodistomum lohrenzi* (Loewen, 1935) and *Catoptroides lacustri* Loewen, 1929, of the subfamily GORGODERINAE. In obtaining this abundance of living material we have been able to work out the details of the excretory system for these species. In the present paper, therefore, we propose to present a description of the complete excretory system for each of the three species and make pertinent comments on the validity of the genera concerned.

THE EXCRETORY SYSTEM IN GORGODERINAE

The excretory pore (EP), Figs. 1–4, is located terminally or slightly dorsal to the posterior extremity of the body, depending on the amount of contraction in that region of the body at the time of fixation. It appears as a small indentation on the surface and leads inward for a short distance to a small, although well developed sphincter muscle (S) (not mentioned for *Gorgoderina tanneri*). Beyond the sphincter the bladder (EB) continues inward, gradually passing dorsally as it makes its way anteriorly to about the level of the ovary. In the area adjacent to the sphincter, the bladder is often slightly dilated, and depending on the amount of contraction in the posterior part of the body and on the amount of waste material in the bladder, there may be more than one such dilatation, although the farther away from the sphincter the less pronounced are these secondary dilatations (mentioned by Looss, 1894). With an average amount of contraction in the posterior part of the body, the bladder is slightly dilated adjacent to the sphincter and the remainder is a more or less cylindrical tube with few or no irregularities except where it curves right or left, dorsally or ventrally, around the internal organs.

The common collecting tubules (CT) emerge from the bladder just posterior to its anterior termination in *Gorgodera*, *Phyllodistomum*, and *Catoptroides* while the bladder gives rise to two short, slightly dilated cornua (CO) in *Gorgoderina*. In the former case the bladder forms a small, knot-like bulb (N) anterior to the point of origin of the common collecting tubules. In the latter case the short cornua give rise to the common collecting tubules from their extremities.

The common collecting tubules (CT) usually make a wide sweep ventrally and laterally and pass underneath the ovary in reaching their positions in the lateral margins of the body. They have been observed to follow a course outward posterior to the ovary (O), over the ventral face of the ovary, or posterior to the ovary. When the testes are located far enough forward to be on a level approximating that of the ovary, the common ducts may diverge laterally so as to pass ventrally to these organs. In all cases the common tubules appear to be more or less straight ducts that curve slightly anteriorly in passing outward from the bladder to near the lateral margins of the body where they give rise to the anterior (M_1) and posterior (M_2) main collecting tubules (*Gorgoderina* and *Phyllodistomum*) while in *Gorgodera* and *Catoptroides* these same ducts become greatly convoluted after passing antero-laterally to near the sides of the body and then continue anteriorly to the level of the bifurcation (BF) of the ceca (CE) before giving rise to the two main collecting tubules.

In all of the material studied by us, each of the main collecting tubules gives rise to four accessory tubules, the anatomical details of which are

given in the description of the excretory system for each species. In every instance, each accessory tubule gives rise to four capillary tubules and each of these terminates in a single flame cell. The flame cell formula, as determined for our material, confirms the observation made by Olsen (1937) on the flame cell pattern of *Gorgoderina tanneri,* the type of which was $2 \times 8 \times 4 = 64$ flame cells.

Gorgodera amplicava Looss, 1899

(Fig. 1)

The sub-cylindrical bladder (EB) is surrounded by a small, well developed sphincter (S) at the pore (EP) and there is a small, knot-like bulb (N) anterior to the origin of the common collecting tubules (CT). The common collecting tubules are evenly curved as they swing outward and upward from the bladder to a point approximating the posterior margin of the acetabulum; then they become greatly convoluted and pass forward to the region of the bifurcation (BF) of the ceca (CE). At this point each of the common tubules is reflexed for a short distance before it bifurcates (I) into the two main collecting tubules. The anterior main collecting tubule (M_1) passes posteriorly and laterally from the bifurcation through a convoluted course and gradually becomes smaller and smaller as it continues to give off its four accessory tubules. Ultimately it terminates at about the level of the anterior margin of the large acetabulum (VS). The first accessory tubule (A) emerges from its main tubule a short distance from the junction of the two main tubules and proceeds forward to the level of the oral sucker where it gives rise to four capillaries, each of which terminates in a flame cell. This accessory tubule (A), as is the case with all of the accessory tubules, gives rise to three capillaries at its termination and the fourth capillary originates a short distance before the termination. The second accessory tubule (B) emerges from the main tubule a short distance from the origin of the first and passes forward to about the level of the bifurcation of the ceca before giving rise to its characteristic group of four capillaries and four flame cells. The third (C) and fourth (D) accessory tubules originate simultaneously near the level of the anterior margin of the acetabulum, thus obliterating the anterior main tubule. The third accessory tubule (C) passes inward toward the midline before giving rise to its four capillaries and flame cells and the fourth accessory tubule (D) proceeds posteriorly to the level of the equatorial plane of the acetabulum where it gives rise to its group of four capillaries and flame cells; the most posterior flame cell of this group lies near the caudal margin of the acetabulum.

The posterior main collecting tubule (M_2) is a greatly convoluted duct that passes posteriorly from its origin (I) to near the posterior margin of the testicular mass. In the anterior part of the body this tubule often crosses the anterior main tubule and common ducts so that they appear as a confused mass of ducts lying near the margin of the body from the level of the bifurcation of the ceca to the posterior boundary of the acetabulum. The first accessory tubule (D_1) originates at about the level of the ovary and passes forward to the region of the vitelline bodies before it gives rise to its group of four capillaries and flame cells. The second accessory tubule (C_1) arises caudal to the level of the anterior margin of the testis and passes anteriorly for a short distance to give rise to its four capillaries and flame cells. The third (B_1) and fourth (A_1) accessory tubules originate together at a level approximating one-third the length of the testis from the posterior margin of that body; the third passing anteriorly for a short distance and the fourth proceeding on posteriorly to about the caudal boundary of the testis. Each of these accessory tubules gives rise to four capillaries and flame cells.

The flame cell pattern is of the $2 \times 8 \times 4 = 64$ type.

Catoptroides lacustri Loewen, 1929

(Figs. 2 & 2-A)

The excretory system of *Catoptroides lacustri* is identical with that described for *Gorgodera amplicava* as outlined above except for a few minor differences which will be discussed.

Although the bladder is not pouched nor does it depart from the general pattern outlined for the group, it does show a very irregular wall from the pore to the anterior extremity. In the material examined by us there is not so conspicuous a dilation in the bladder immediately anterior to the sphincter as in *Gorgodera amplicava,* and the bladder ends a little in advance of the ovary.

The common collecting tubules (CT) are of the same general pattern as in *Gorgodera amplicava,* while the main collecting tubules (M_1 & M_2) differ from those of the previous species in that they divide into secondary tubules before giving rise to the accessory tubules. Each anterior main tubule (M_1) branches (II) into a pair of secondary tubules (X & Y). One of these branches (X) passes outward through a convoluted course to near the lateral margin of the body where it gives rise (1) to two accessory tubules. One of these accessory tubules (A) proceeds anteriorly to the region of the oral sucker, and the second one (B) passes posteriorly for a short distance; each accessory tubule terminates in a group of four capillaries and flame cells. The other secondary tubule (Y) passes posteriorly through a sinuous course to a level midway between the ventral sucker and genital pore where it bifurcates (2) into two accessory tubules. One of these (C) courses outward toward the margin of the body and gives rise to its group of four capillaries and flame cells, while the other one (D) proceeds directly posteriorly to the region immediately caudal to the anterior margin of the acetabulum before forming its group of four capillaries and flame cells.

The posterior main collecting tubule (M_2) passes posteriorly from its origin (I) to near the level of the ovary before branching. The first of its secondary branches (X_1) is very short, giving rise immediately to two accessory tubules. These two accessory tubules diverge, one (D_1) passing anteriorly and the other (C_1) posteriorly for relatively short distances before forming their complement of four capillaries and flame cells each. The other secondary branch (Y_1) of the posterior main tubule is relatively long, passing posteriorly to about the level of the caudal boundary of the testis before giving rise to its two accessory tubules. One of these (B_1) passes mesiad for a short distance and the second one (A_1) proceeds posteriorly to near the end of the body. Each of these ducts gives rise to the characteristic number of four capillaries and flame cells.

The flame cell pattern is of the $2\times8\times4=64$ type.

Phyllodistomum lohrenzi (Loewen, 1935)

(Fig. 3)

The structure and configuration of the excretory bladder in *Phyllodistomum lohrenzi* is identical with that described for *Gorgodera amplicava.*

The common collecting tubules (CT) diverge antero-laterally from the bladder and each extends forward only as far as the posterior level of the acetabulum. At this level each common tubule bifurcates (I) into anterior (M_1) and posterior (M_2) main collecting tubules. The anterior main collecting tubule passes forward through a convoluted course to near the oral sucker, giving rise to the accessory tubules along its course. One of these (D) emerges at about the level of the anterior margin of the acetabulum, another (C) at about the level of the genital pore, and two (B & A) a little in advance of the bifurcation of the ceca. The posterior main collecting tubule (M_2) passes posteriorly and becomes exhausted near the caudal margin of the body. Along its course this tubule gives rise to four accessory tubules; one (D_1) near the level of the ovary, one (C_1) at about the anterior level of the caudal testis, and two (B_1 & A_1) near the posterior level of the same testis.

Each accessory tubule gives rise to four capillaries in the manner described for

these tubules in *Gorgodera amplicava* and each capillary terminates in a single flame cell.

The flame cell pattern is of the $2 \times 8 \times 4 = 64$ type.

Gorgoderina tanneri Olsen, 1937

(Fig. 4)

The excretory system of *Gorgoderina tanneri*, as worked out by Olsen (1937), differs from that described for *Phyllodistomum lohrenzi* in the following respects: 1. No mention is made of the presence or absence of a sphincter muscle guarding the pore. 2. The bladder gives rise to two short, slightly dilated cornua from its anterior end, and the cornua give rise to the common collecting tubules from their anterior extremities. 3. The bladder ends before the level of the ovary is reached. 4. The common collecting tubules diverge almost laterally from the point of their origin and bifurcate into anterior and posterior main collecting tubules at about the level of the caudal boundary of the ovary. 5. The anterior main collecting tubule gives rise to four accessory tubules, of which only the most anterior one is as long as in *Phyllodistomum lohrenzi*. 6. The two most anterior accessory tubules with their groups of capillaries and flame cells are located anterior to the level of the ventral sucker, while the other two accessory tubules, originating from the anterior main tubule, with their capillaries and flame cells are located behind the level of the acetabulum. 7. There is a decided tendency for the capillaries and flame cells originating from the accessory tubules of the posterior main tubule to be crowded into the extreme posterior portion of the body.

DISCUSSION

In the present paper we have described the details of the excretory system for representative species, *Gorgodera amplicava* Looss, 1899, *Catoptroides lacustri* Loewen, 1929, *Phyllodistomum lohrenzi* (Loewen, 1935), and *Gorgoderina tanneri* Olsen, 1937, of four genera belonging to the subfamily GORGODERINAE. The details of this system are described for the first time for the first three species, the system of the fourth species having been described by Olsen (1937). It is included in the present paper for comparison.

A casual comparison of the illustrations is sufficient to convince one that there are marked differences in the morphological details of the excretory systems in the four species under consideration. When studied carefully these differences are beyond all expectations for so conservative a system as that of excretion in the trematodes. On the basis of our present knowledge it is customary for the student of the excretory system to expect this system to conform to a well-described pattern in closely related species as would comprise a single genus. It is not at all surprising to find that this same pattern, with or without slight modifications, remains constant among closely related genera, such as the genera assigned to a natural subfamily or even a family. Any definite departure from the pattern characteristic for a well-established group immediately leads one to the conclusion that the species or genus showing such a departure must have affinities elsewhere.

In considering the representatives of the four genera, described above, as a group, we are impressed by the uniformity in the fundamental flame

cell pattern. Contrary to the contention made by Looss (1894) and later somewhat confirmed by Sturges (1897), there is a definite and constant number of accessory tubules, 8 in number, 4 from each main tubule, on each side of the body instead of 12 as given by Looss or an inconstant number as indicated by Sturges. Each of the accessory tubules gives rise to four capillaries, each of which is provided with a single flame cell. In determining the total number of flame cells in *Gorgodera cygnoides,* Looss (1894) recorded the presence of 12 accessory tubules on each side of the body and these gave rise to an inconstant number of capillaries, of which four was the smallest and eight the largest number. Then taking an average of this inconstant number, Looss gave the probable total number of flame cells as $2 \times 12 \times 6 = 144$. Regarding the number of capillaries and flame cells, more recent findings (Olsen, 1937, and the present paper) show a constant number originating from a total of 16 accessory tubules, 8 on each side of the body. Thus, in consideration of the total number of accessory and capillary tubules and flame cells all four of our representative species from as many genera of the subfamily GORGODERINAE exhibit a remarkable uniformity of pattern.

When we consider the length and nature of the common collecting tubules, the manner in which they unite with the bladder, and the way in which the main collecting tubules give origin to the accessory tubules, it is then that we are impressed with the differences found in the excretory systems of this group of worms. From the standpoint of the tubular system, we find our representative species becoming differentiated into two subgroups: 1. The *Gorgodera-Catoptroides* group in which the common collecting tubules are long, reaching up to the bifurcation of the ceca before giving rise to the anterior and posterior main collecting tubules. 2. The *Phyllodistomum-Gorgoderina* group in which the common collecting tubules are short, reaching only to near the margins of the body and giving rise to the anterior and posterior main collecting tubules posterior to the level of the ventral sucker.

In the first group, *Gorgodera-Catoptroides,* a much more elaborate system of tubules is to be met with than is the case with the latter group. The common collecting tubules (CT) in *Gorgodera* and *Catoptroides* emerge from the bladder a short distance behind the anterior extremity of the vesicle and proceed forward through a convoluted course, near the lateral margins of the body, to the level of the bifurcation of the ceca, there becoming reflexed before branching into the two main collecting tubules. The anterior main collecting tubule (M_1) on each side of the body proceeds posteriorly to about the level of the ventral sucker before it is exhausted. The first accessory tubule (A) of this main duct is given off nearest the junction of the two main tubules and turns anteriorly to give rise to capillaries and flame cells in the region of the oral

sucker. Whether or not this accessory tubule is the primary one is beside the point, and could be determined only through a study of the formation of the excretory system in the developmental stages from the cercarial germ mass to the fully matured individual. The other three accessory tubules of the anterior main tubule are given off in a manner not radically unlike that in other trematodes.

The posterior main collecting tubule (M_2) proceeds posteriorly through a much convoluted course from the bifurcation (I) to near the caudal end of the body, giving rise to accessory tubules in a more orthodox manner.

From the standpoint of genera, *Catoptroides* differs from *Gorgodera* in the arrangement of the tubules branching off from the main tubules (notwithstanding differences in general morphology of the body). Each of the main collecting tubules divides into two secondary tubules (Fig. 2–A) and from each of the secondary branches arises two accessory tubules. The two secondary branches of the anterior main collecting tubule are of about equal length, while the two secondary branches originating from the posterior main collecting tubule are quite unequal in length, one giving rise to its two accessory tubules immediately after emerging from the main tubule. The other secondary tubule equals or exceeds the length of the secondary branches of the anterior main tubule. Thus, *Catoptroides* differs from *Gorgodera* in that the accessory tubules take their origin from secondary collecting tubules instead of from the main collecting tubules as is the case in the latter genus.

Within recent years students of this subgroup (Gorgoderinae) of trematodes have found it extremely difficult to reach an agreement as to the validity of the genus *Catoptroides* since the characters on which the genus was founded are of such an "inconsequential and variable" nature (Lynch, 1936) and due to the presence of intermediate and intergrading forms (Lewis, 1935, and Lynch, 1936). These observations tend to confirm the conclusion that *Catoptroides*, in the light of more recent knowledge of *Phyllodistomum* (Nybelin, 1926), cannot be considered sufficiently distinct from *Phyllodistomum* to warrant its retention for those species now assigned to it. The elucidation of the details of the excretory system for one member of the genus, *Catoptroides lacustri*, should be of aid in establishing the validity of the genus. A study of the details of the excretory system in the representative member of the genus convinces us that *Catoptroides* is more closely related to the genus *Gorgodera* than to the genus *Phyllodistomum*.

In the second group, *Phyllodistomum-Gorgoderina*, we find more or less uniformity in the excretory system. The two genera differ from one another in regard to the anterior end of the bladder and the distribution of the groups of capillaries and flame cells. In the genus *Phyllodisto-*

mum the common collecting tubules are joined to the bladder a very short distance behind its anterior end, while the bladder in *Gorgoderina* terminates by giving rise to two short cornua, from the ends of which spring the common collecting tubules. In the genus *Phyllodistomum* the accessory tubules and their capillaries with flame cells are rather evenly distributed along the course of the main collecting tubules, whereas in the genus *Gorgoderina* two of the accessory tubules with their capillaries and flame cells of the anterior main tubule are located in the fore-body anterior to the acetabulum and the other two accessories with their groups of capillaries and flame cells of the same duct are placed in the body posterior to the ventral sucker.

We are in agreement with Pande (1937) in considering the shape of the fore- and hind-body in members of the subfamily GORGODERINAE to be of insufficient value as a character on which to base the description of a genus. We are in agreement also with the view expressed by Steen (1938) when this author considered Pande's reasons for declaring *Gorgoderina* a synonym of *Phyllodistomum*. Steen (p. 205) states: "This is open to question and will be discussed in a later paper." Based on the present study of this group of worms, we are of the opinion that the genus *Gorgoderina* must stand as valid until more information concerning detailed anatomical and life history studies on additional species belonging to the several genera now included in the subfamily is made available.

On the basis of a study of the details of the excretory system in this representative group of species belonging to four of the genera now assigned to the subfamily GORGODERINAE the present indications are that the subfamily is an unnatural one and will have to be revised when more material is available for detail study. It seems to us to be unwise at the present time to conclude from our limited knowledge of the group that other variations in the structural make up of the excretory system will not be met with when other members of the group are studied. At the present time, however, the demonstrated variations that exist in the excretory systems of the representative species, belonging to four of the genera of the subfamily group, most certainly suggest a very close relationship between the genera *Gorgodera* and *Catoptroides* on the one hand and between *Phyllodistomum* and *Gorgoderina* on the other. If these same variations are found to be constant among the members of the various genera, when such information is made available, it would seem to us we are dealing with a group of worms of less compatibility than ordinarily would be grouped into the same subfamily.

SUMMARY

A brief review of the status of the subfamily GORGODERINAE is presented. In doing this it is our purpose to direct attention to the genus

Catoptroides since it is this genus that previously has been too poorly defined to warrant generic distinction.

The details of the excretory system for the species *Gorgodera amplicava* Looss, 1899, *Catoptroides lacustri* Loewen, 1929, and *Phyllodistomum lohrenzi* (Loewen, 1935) are presented for the first time. The details of the excretory system of *Gorgoderina tanneri* Olsen, 1937, are included for comparison, the system having been worked out by Olsen (1937).

Based on a study of the excretory system alone, the genus *Catoptroides* appears to be quite distinct from the genus *Phyllodistomum,* the genus with which it is most often confused, and is shown to be more closely related to the genus *Gorgodera* than is the case with either of the other genera studied.

The question of *Gorgoderina* becoming a synonym of *Phyllodistomum* is an open one, and will be determined only when more information is made available concerning detailed anatomical and life history studies.

It is suggested that when more information on the details of the excretory system in the other members of the genera now assigned to the subfamily group is available it may become necessary to revise the subfamily, dividing it into two subfamilies.

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Explanation of Plates

I = bifurcation of common collecting tubule into anterior and posterior main collecting tubules
II = bifurcation of anterior main collecting tubule into secondary collecting tubules
1 = bifurcation of anterior secondary collecting tubule into accessory collecting tubules
2 = bifurcation of posterior secondary collecting tubule into accessory collecting tubules
A = first accessory collecting tubule
A_1 = eighth accessory collecting tubule
B = second accessory collecting tubule
B_1 = seventh accessory collecting tubule
BF = bifurcation of ceca
C = third accessory collecting tubule
C_1 = sixth accessory collecting tubule
CE = cecum
CO = cornua of bladder
CT = common collecting tubule
D = fourth accessory collecting tubule
D_1 = fifth accessory collecting tubule
EB = excretory bladder
EP = excretory pore
ES = esophagus
F = flame cell
GP = genital pore
M_1 = anterior main collecting tubule
M_2 = posterior main collecting tubule
N = anterior knot-like bulb terminating the excretory bladder
O = ovary
OS = oral sucker
S = sphincter muscle
T = testes
T_1 = anterior testis
T_2 = posterior testis
V = vitellaria
VS = ventral sucker
X = anterior secondary branch of the anterior main collecting tubule
X_1 = anterior secondary branch of the posterior main collecting tubule
Y = posterior branch of the anterior main collecting tubule
Y_1 = posterior branch of the posterior main collecting tubule

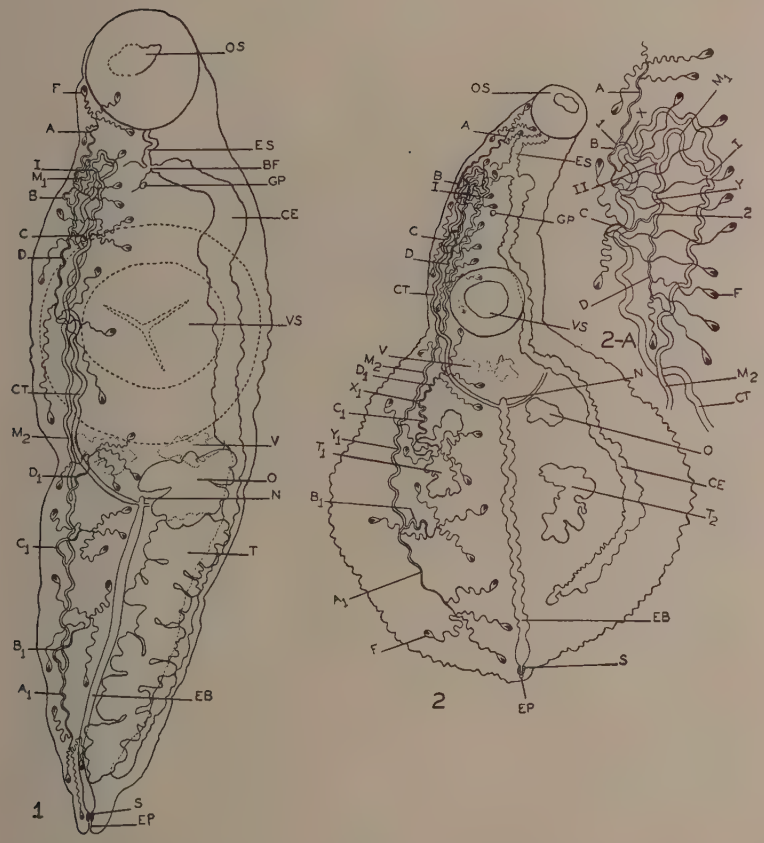
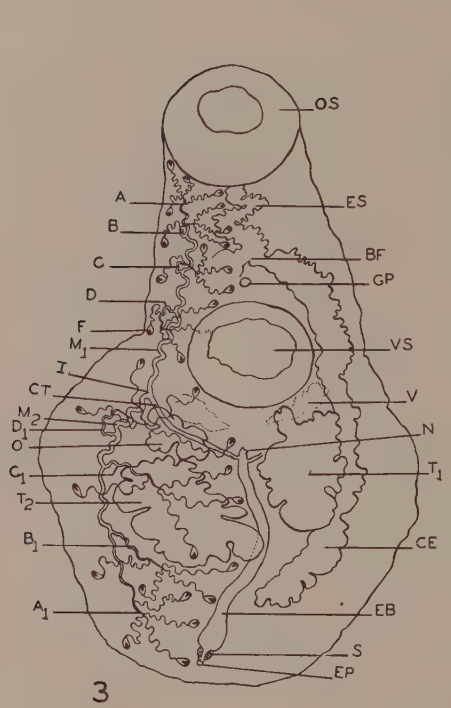


PLATE I

FIG. 1. The excretory system of *Gorgodera amplicava* Looss, 1899, with details omitted from the right side. Dorsal view. Original.

FIG. 2. The excretory system of *Catoptroides lacustri* Loewen, 1929, with details omitted from left side. Ventral view. Original.

FIG. 2-A. Diagrammatic view of the tubules of the excretory system in the left side of the anterior part of the body of *Catoptroides lacustri* Loewen, 1929.



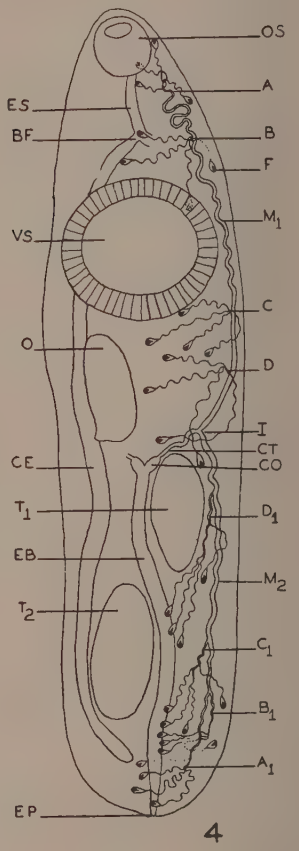


PLATE II

FIG. 3. The excretory system of *Phyllodistomum lohrenzi* (Loewen, 1935), with details omitted from the left side. Ventral view. Original.

FIG. 4. The excretory system of *Gorgoderina tanneri* Olsen, 1937, with details omitted from the right side. Ventral view. After Olsen.

THE EFFECTS OF MONOCHROMATIC ULTRAVIOLET RADIATION ON EGGS OF THE NEMATODE, *ENTEROBIUS VERMICULARIS*. I. QUANTITATIVE RESPONSE

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There exists an extensive literature on the effect of ultraviolet radiation on eggs of invertebrates. Good reviews of this work are available in "Biological Effects of Radiation" (Duggar, 1936). Most of this work used the entire radiation from a mercury vapor lamp or broad regions separated by filters. Only very little material is available where monochromatic radiation has been used. A number of papers appeared 20 to 30 years ago which took advantage of the ultraviolet microscope (Köhler, 1904) and used wavelength 2800Å, which was made available by this instrument (Stevens, 1909, Schleip, 1923).

This report will discuss the effects of monochromatic ultraviolet radiation between the wavelengths 2280 and 3150Å on the eggs of *Enterobius vermicularis,* the common pinworm infecting man. This study was initiated in view of the increasing interest in the use of ultraviolet radiation as a killing agent for bacteria and fungi, and in order to investigate the value of such radiation as a killing agent for pinworm eggs. These eggs are not only scattered over floors, bedding, etc., in infected households and in institutions, but are probably airborne to some extent and may be dispersed about homes, schools, and playgrounds, thus constituting a definite public health hazard (Oleinikov, 1929; Lentze, 1935; Cram, Jones, Reardon and Nolan, 1937; Nolan and Reardon, 1939). Satisfactory measures to control the spread of infection and to prevent reinfection of treated persons are needed and suitable killing agents for the scattered eggs are being sought.

A number of studies report on the effects of radiation on eggs of several species of nematodes (Wright and MacAllister, 1934; see also for literature). The only previous report concerning ultraviolet irradiation of this nematode, *Enterobius vermicularis,* known to us is that of Shalimov (1935). Shalimov used a commercial mercury vapor lamp as a source of radiation and tested eggs with embryos not yet developed to an infective stage; she suggested that ultraviolet radiation might be of especial importance in efforts to control pinworm infection.

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EXPERIMENTAL TECHNIQUE

Treatment of Eggs before Irradiation

Migrating female pinworms collected by clinic patients were the main source of the eggs. Some worms which were collected from stool samples were also used. These latter were living females which oviposited when transferred into a clean dish. Care was taken to use eggs from only one worm in an experiment. The worms or eggs thus collected were transferred into clean Syracuse watch glasses containing distilled water; generally only the eggs which floated after being thus transferred were used. Most of the eggs were still in the tadpole stage when received and were incubated at 37° C for a minimum of 5½ hours until they reached the infective stage. In a few instances when material was received late in the day it was incubated overnight. Eggs were checked for the presence of active "ring-and-a-half" or infective stage embryos. In addition, a sample of 150 to 200 eggs was tested for ability to hatch in artificial gastric juice (0.7 per cent HCl and 0.5 per cent pepsin); it was found that unless control dishes hatched at least 80 per cent active larvae at the beginning of the experiment, the material was not satisfactory. Eggs varied in age from 3½ to 8 days at beginning of the experiments. Material was kept in a refrigerator (3 to 5° C) until used.

The individual dishes for irradiation were prepared in the following manner: Small Syracuse watch glasses, 20 mm in diameter, were thoroughly cleaned, and 0.4 cc of distilled water was placed in them. Eggs were then transferred to the dishes by means of a small bamboo needle, about 150 eggs being placed in each watch glass. The dishes were then ready for irradiation. Dishes to be used as controls were handled in a manner identical with that of the exposed dishes.

Irradiation Technique

The eggs of this nematode (see Fig. 1) are about 50 to 60 μ long by 20 to 30 μ wide, and are covered by a shell consisting of three layers composed, respectively, of protein, chitin, and lipoid. The shell has a small

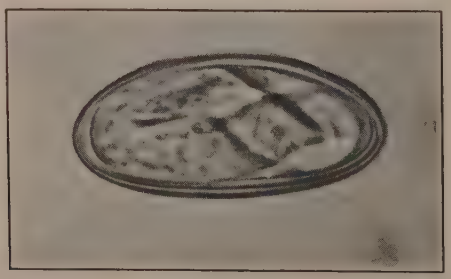

FIG. 1. Photograph of a pinworm egg in the ring-and-a-half stage.

area towards the tip, the hatching area, which is covered only by layers of protein and lipoid. Insofar as possible, in our study we irradiated the eggs from all sides in an attempt to obtain uniform distribution of radiation over the entire egg. It was also necessary to be certain that each egg in an exposed dish received an equivalent amount of energy in order that the statistical interpretation of the results obtained on a large number of eggs be valid.

A technique which we developed in connection with the study of the effects of monochromatic ultraviolet radiation on the eggs of *Arbacia punctulata* (Hollaender, 1938) seemed to be suitable for this investigation. The eggs of *Enterobius vermicularis* were not available to us in such large numbers as *Arbacia* eggs and also are not as suitable material for biophysical investigation as those of *Arbacia* because the pinworm eggs have a slight tendency to clump and stick to glass. The technique finally used was a slight modification of the technique described in the previous article. An intense beam of radiation from a vertical water-cooled high-pressure quartz capillary mercury vapor lamp (Daniels and Heidt, 1932) was concentrated on the entrance slit of the crystalline quartz monochromator. The emerging monochromatic beam was made to cover by means of a right angle quartz reflecting prism and a quartz condensing lens, an area of 2×14 mm, or a vertical area of the size of the receiving surface of a high sensitivity thermopile. The change from a horizontal to vertical area could be made easily by a 90° turn of the reflecting prism. The thermopile was connected to a high sensitivity Leeds and Northrup galvanometer and standardized against a National Bureau of Standards "standard lamp."

Two dishes containing eggs were fastened to two rotating platforms; one dish to be placed under the reflection prism and exposed to radiation; the other dish to serve as a control but rotated in the same manner as the exposed dish. The only differences between the method used earlier for *Arbacia* eggs and that of the present experiment were that the dishes used for the pinworm eggs were smaller in diameter, and the eggs were stirred by an air stream by hand and not mechanically as with *Arbacia*.

The effect of seven wavelengths was investigated, i.e., 2280, 2380, 2480, 2537, 2650, 2806, and 2950Å. A few experiments were conducted with wavelengths above 3000Å but, since no effect was found with the energies used in this study, these wavelengths longer than 3000Å were not tested further in our experiments. About five exposures were made for each wavelength in each set of experiments.

The criteria available to us for measuring the extent of injury to pinworm eggs by irradiation were more limited than in the case of those eggs which are not embryonated when laid and in which further development can be used for determinations of experimental effects. It is diffi-

cult to control irradiation experiments with the pinworm egg especially when it is in the tadpole stage. The tadpole-stage embryo present in the eggs when laid rapidly develops to the infective ring-and-a-half stage which is more resistant to unfavorable environmental conditions (Zawadowsky and Shalimov, 1929). Therefore, with the exception of a few experiments designed to test the effect of radiation on the further development of tadpole-stage eggs which will be reported in a second article, our work was confined to studies on the effects of radiation on the ring-and-a-half stage or infective eggs. These, of course, presented no opportunity for measuring further development since the next stage is parasitic and no hosts other than man are known. Other than ability to hatch the only criterion for determining the effects of radiation was the activity of the embryo within the egg, and this was at best a very unsatisfactory yardstick. Consequently, the ability of the radiated eggs to hatch active larvae, as compared with unirradiated controls, was used to measure the radiation effects.

After irradiation, the eggs were tested at once if possible; otherwise immediately after being irradiated they were again refrigerated at about 5° C for a short time. For testing the ability to hatch, the dishes were placed in a moist chamber at 37° C for 30 minutes, and then 0.6 cc of digestive juice (0.7 per cent HCl and 0.5 per cent pepsin) was added. After the ova had been allowed to remain in the incubator for another 45 minutes, they were examined for larvae. The examination was made with a dissecting microscope (10 × objective and 12.5 × ocular) with the Syracuse glass on a warm stage kept at approximately 37° C. All active larvae found were removed and counted. The dish was then again incubated and examined at 50-minute intervals until a total of four hours had elapsed from the time digestive juice was added. The number of active larvae hatched from the eggs was divided by the total number of eggs in the dish at the start of the experiment to give the percentage of active hatch or the per cent survival of the irradiated eggs in R dishes and, similarly, the per cent hatch of the control eggs in C dishes. The per cent survival of the irradiated eggs was divided by that for the respective controls to give a corrected figure for survival of irradiation which we express in per cent and designate as the R–C survival ratio. The remaining percentage, i.e., of 100 per cent, represents the per cent inactivation actually due to irradiation. Tables 1 and 2 give the final total values of eggs hatching living larvae.

Before the above technique was adopted, a saline solution was used instead of distilled water; digestive juice was added to the dishes as soon as they were placed in the incubator, and no time limit was used; the dishes were examined at intervals until no further hatching was observed. Since evaporation took place to varying degrees during irradiation and

TABLE 1.—*Energy response of inactivation at wavelength 2280Å*

Experiment No.	Exposure minutes	Galvanometer deflection	Incident energy in ergs/per egg	Per cent hatched	R–C survival ratio	Per cent inactivation
R 1	8	8.7 cm	1.0	60	73	27
R 2	17	6.3 cm	1.5	24	28	72
R 3	36	5.0 cm	2.5	13	16	84
R 4	60	5.0 cm	4.1	10	12	88

Column 5 gives per cent eggs hatched per dish exposed; column 6, per cent survival of radiated eggs compared to controls (see p. 424); column 7, the percentage inactivation for which the irradiation is responsible.

TABLE 2.—*Wavelength dependence for fifty per cent inactivation*

Wavelength in Ångströms	January 15, 1939		March 5, 1939	
	Incident energy in ergs/egg	Relative efficiency	Incident energy in ergs/egg	Relative efficiency
2280	1.4	10.0	5.3	10.0
2380	2.3	6.1	7.1	7.5
2480	...	...	2.0	2.6
2527	5.5	2.5	...	...
2650	9.0	1.6	2.0	1.9
2805	4.1	3.6	22.0	2.3
2950	9.7	1.4	47.0	1.1

Columns 3 and 5 give the relative efficiency taking the results at 2280Å as 10.0.

while the dishes were on the warm stage under the microscope the saline solution became more concentrated and appeared to influence hatching; therefore, its use was discontinued. In the procedure adopted later, the 30-minute interval between the time the dishes were placed in the incubator and the time that digestive juice was added to them was necessary to equalize differences in the temperature of various dishes, according to the way they had been handled before incubation; i.e., whether they had been kept at room temperature or refrigerated.

The time involved in examination limited the number of irradiated dishes that could be tested in one day, and also the number of control dishes that could be used.

Active larvae which hatched during a four-hour period, when tested after irradiation, were considered as having survived irradiation. It was found often that some larvae which had hatched from eggs surviving a considerable amount of radiation could live outside the egg shell only for a relatively short time as compared with larvae from controls. Besides this weakening of hatched larvae we observed other secondary effects, such as a delay in hatching, and a delayed lethal action. This matter will be discussed in a subsequent paper (Jones, Jacobs, and Hollaender).

RESULTS

Data presented in this report are based on observations on 97 irradiated dishes and 36 controls in which distilled water was used. These dishes represent 9 different batches of eggs. Observations on 110 irradi-

ated dishes and about 40 controls in which saline was used were available for comparison.

The energy received by each egg was calculated in the following manner. The following factors were known: Size of the egg; size of the dish; size of the monochromatic beam; and intensity of the beam. The following formula was therefore used:

$$\frac{E \times T \times C \times f}{a} = I$$

For a typical experiment:

E = Incident energy in ergs per second = 67.5 (ergs/cm/second) × 8.75 (cm deflection)

T = Time of exposure = 8×60 seconds

C = Cross sectional area of the egg = $1180\mu^2$

f = Fraction of time each egg is exposed = $\frac{1}{12.3}$

a = Area of beam = $28 \times 10^6\mu^2$

I = Incident energy per egg

$$\frac{67.5 \times 8.75 \times 8 \times 60 \times 1180}{28.6 \times 10^6 \times 12.3} = .98 \text{ ergs per egg.}$$

This calculation was justified because the eggs were rotated evenly and came into the monochromatic beam with great uniformity since no

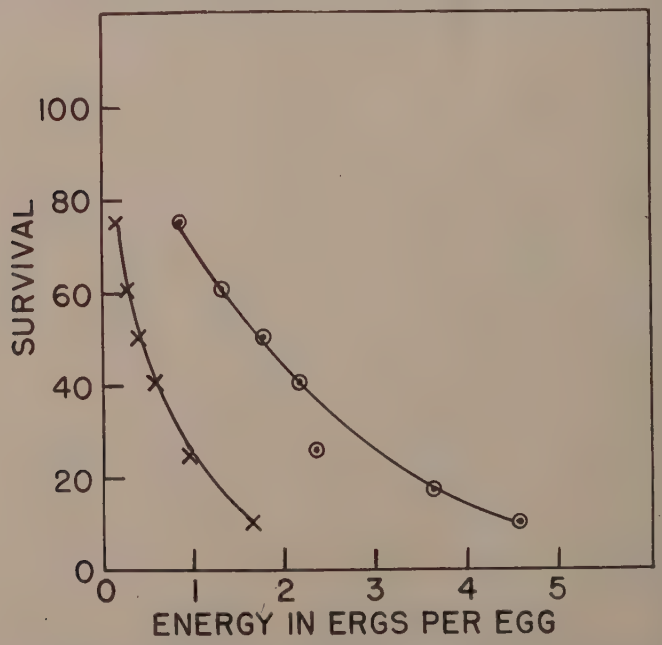

FIG. 2. R–C survival ratio plotted against incident energy in ergs/egg for 2280Å (X) and 2650Å (O). Based on averages of plotted data of several experiments.

crowding took place. Typical results are given in Table 1, the data on one wavelength from an experiment with a relatively resistant lot of eggs.

Characteristic curves of the killing effect of two wavelengths are given in Fig. 2, in which the incident energy per egg is plotted against the R–C survival ratio; the curves are based on averages of plotted data from several different experiments. Curves were obtained for all the wavelengths tested. As can be seen from this figure, the spread of error is quite considerable. The error was especially great with different batches of eggs obtained from different individuals. Consequently for any comparative tests, as for wavelength dependence tests of killing, only eggs from one individual were used.

Table 2 gives data for 50 per cent inactivation at seven different

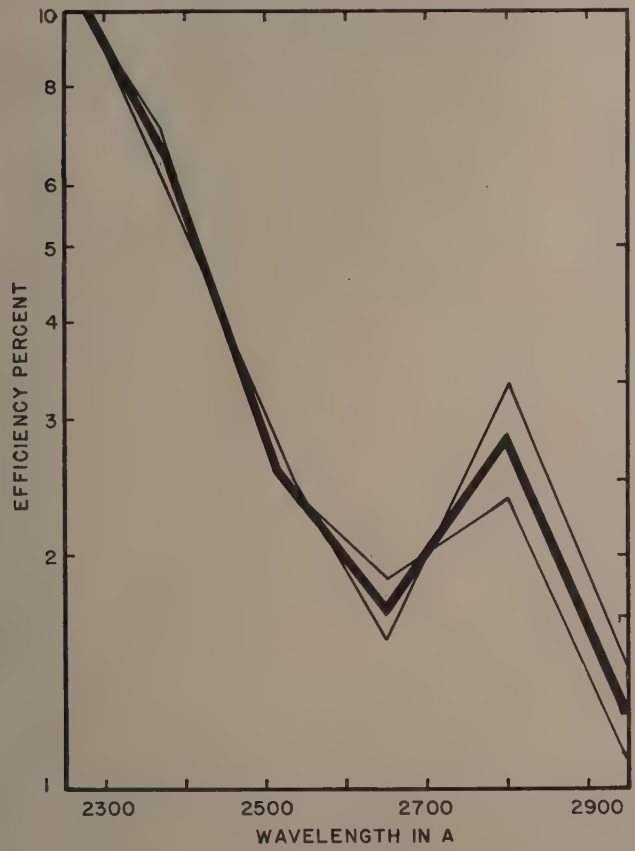

FIG. 3. Relative efficiency for 50 per cent inactivation (taking energy at 2280Å as 10 = 100) plotted against wavelengths. Fine lines represent data for two experiments (see Table 2) and heavy line the medium.

wavelengths obtained for two entirely different batches of eggs. The values of Columns 3 and 5 of this table are plotted in Fig. 3. The heavy line in this figure gives the medium value. This figure gives only the relative efficiency of the different wavelengths for 50 per cent inactivation; for absolute energy values, Table 2 should be consulted.

DISCUSSION

In any discussion of the effects of measured quantities of monochromatic ultraviolet radiation, one should emphasize first the limitations of the technique of exposing each individual egg to an equivalent amount of monochromatic radiation. Our method of rotating the exposed dish and of blowing the stream of air against the surface insured that all free, floating single eggs received on the average an equivalent amount of energy during each exposure. However, occasionally a few eggs would stick to the rim of the dish and could not be reached by the radiation; also, occasionally there would be a few eggs which would stick together and protect each other against the full extent of the radiation. In any case, these two factors are not considered a serious handicap to our study since they usually involved only a small percentage of the eggs. However, these two sources of error may account for the fact that it is extremely difficult to kill or inactivate the last few per cent of the eggs. We are showing in Fig. 2 survival ratios up to 90 per cent. Survival ratios below this were erratic and are not shown.

Another factor possibly contributing to this may involve the presence in each lot of a few very resistant eggs. Such a condition would have to be expected from a general distribution curve. But at present we feel that the technical difficulties are a greater source of error than the distribution of sensitivity.

We observed besides this variability in response of eggs of individual batches, a very striking variation in response of batches of eggs coming from worms from different patients. This variation of sensitivity of eggs from different sources was in the most extreme cases 5 to 6 fold, for instance, when it takes at 2650Å one erg to produce fifty per cent inactivation, it would take in other batches as much as 5 ergs to produce the same effect at this wavelength. If we realize the probable variation of the sources from which pinworms come, the nutritional level of the patient, the different type of foods the patient has eaten, the pH of the intestine, the variation of the intestinal flora, and also perhaps of equal importance, the variation in the methods and time of obtaining the eggs and bringing them to the laboratory, this variation in response of eggs from different sources is certainly not surprising (see also Zawadowsky and Shalimov).

The variation of different batches of eggs applies only to the absolute energy of killing at individual wavelengths. It does not apply to the

wavelength dependence of killing. This is shown strikingly in Table 2 and Fig. 3. The values given in this table cover only two experiments. Numerous tests were conducted where two or three wavelengths were tested on one batch of eggs. Invariably, the eggs showed highest sensitivity to the radiation of 2280Å, and a slight maximum at 2805Å.

The egg of *Enterobius vermicularis* is a relatively complicated organism if compared with bacteria or fungous spores. The egg shell consists of three different layers. The outermost layer is protein, the middle layer chitin, and the innermost layer lipoids as previously stated (see Jacobs and Jones, 1939). However, the egg is not entirely surrounded by these three layers. Apparently the chitinous layer is missing at the hatching area. Separate tests have shown that the chitinous layer is not highly absorbent for ultraviolet radiation at the wavelengths used in these tests (Cole and Brackett). We would expect that a large part of the radiation below 2400Å is absorbed by the proteinaceous top layer. Further evidence that something of this sort must have taken place is given by the inactivation curve, which resembles closely the absorption spectrum of a typical protein (Caspersson, 1936), with its small maximum at 2805Å and increased absorption below 2400Å. However, we expect that not all of the radiation is absorbed in the egg shell but probably a considerable percentage of this radiation penetrates to the embryo in the egg. The inhibition of hatching by ultraviolet radiation could be produced first by a hardening of the outside protein layer, second, by a change in the composition of the lipoid membrane, third, by irradiation injuring the embryo within the shell, and fourth, by possible toxic substances formed in the egg by this radiation. The production of toxic substances seems the least likely explanation with the energy values used in these experiments. It would seem from the evidence of the shape of the killing curves that death is probably caused by a combination of several factors and not a single effect produced by irradiation.

A test for these different theories of the possible effect of radiation can be made only by a more detailed study of the response of pinworm eggs which have been irradiated by a considerable amount of radiation but which are not entirely inactivated. Such a study will be discussed in a subsequent publication (Jones, Jacobs, and Hollaender).

The wavelength dependence as shown in Fig. 3 is of particular significance from the practical viewpoint of killing eggs. Radiation sources with great intensity below 2400Å would be most useful yet these are difficult to obtain for practical purposes. An additional limiting factor is that under practical conditions pinworm eggs may be covered with dust and debris which are not penetrated by short ultraviolet radiation, and only the eggs which are exposed directly to the radiation without interference of absorbing substances will be killed readily. Although

ultraviolet radiation is more effective against tadpole stage embryos, our tests on infective stage eggs indicate that ultraviolet radiation in itself could be of only limited value in the control of pinworm infection.

It is of interest to note that since sunlight contains only very little radiation (2950–3000Å) tested in these experiments, relatively short exposures to sunlight could have little lethal effect on eggs due to short ultraviolet alone, unassociated with other factors such as high temperature and drying.

If we compare the wavelength dependence of the energy necessary for fifty per cent inactivation for pinworm eggs, bacteria, and fungous spores, striking differences are observable, Fig. 4. Pinworm eggs are

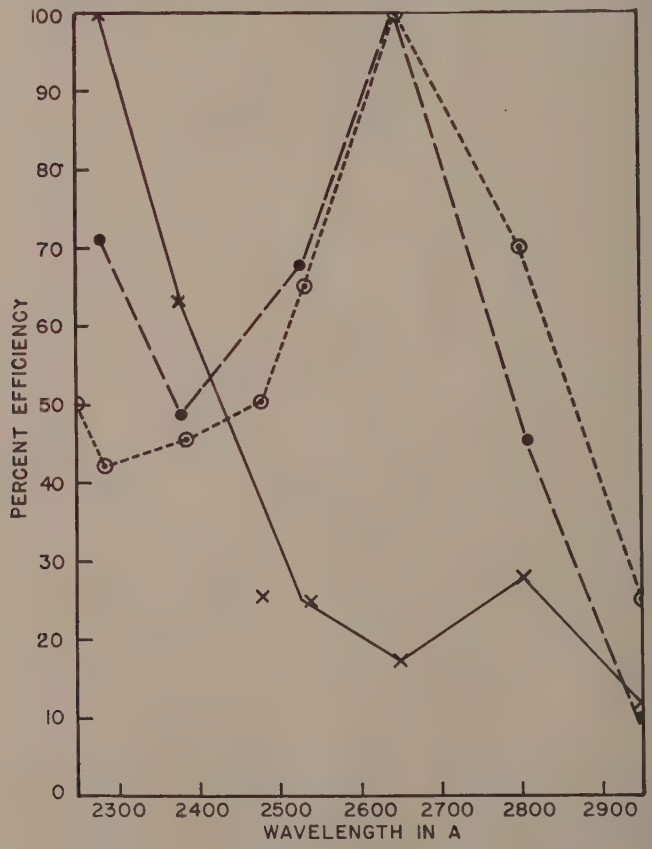

FIG. 4. Comparison of 50 per cent killing curves for inactivation of pinworm eggs (solid), fungous spores (broken), and bacteria (dotted line).

most sensitive at the shortest wavelength tested, i.e., 2280Å, less so at 2650Å and have a slightly increased sensitivity at 2805Å, whereas bac-

teria (Hollaender and Claus, 1936) and fungous spores (Hollaender and Emmons, 1939) have their highest sensitivity at 2650Å. Bacteria and fungous spore inactivation curves resemble nucleic acid absorption curves, whereas the inactivation curves for pinworm eggs resemble closely a protein absorption curve. It takes about one erg (incident energy) at 2650Å to produce fifty per cent killing in pinworm eggs, about 5×10^{-4} ergs (absorbed) to produce fifty per cent killing in fungous spores (dermatophytes) and 5×10^{-6} ergs (absorbed) to produce fifty per cent killing in *E. coli*.

SUMMARY

Technique of preparation of the eggs of *Enterobius vermicularis* for irradiation and the method used in testing results of irradiation are described.

Methods for the efficient irradiation of the eggs with measured quantities of monochromatic ultraviolet irradiation are discussed.

Wavelength dependence of the inactivation of the eggs by ultraviolet radiation from 2280 to 2950Å is covered. This curve shows a small maximum of sensitivity at 2805Å and increased sensitivity at wavelengths below 2400Å.

The theoretical and practical significance of the wavelength dependence curve is discussed.

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RESEARCH NOTES

THE TROPICAL RAT MITE, *LIPONYSSUS BACOTI*, IN MINNESOTA

The growing number of records of occurrence of *Liponyssus bacoti* in the United States serve to emphasize the fact that this species, like the so-called tropical rat flea, *Xenopsylla cheopis,* spreads with its host and may be found established far beyond its supposed range. The following notes on the occurrence of the mite in Minnesota are of interest from this viewpoint.

Some ten years ago the writer was consulted regarding an invasion of minute red "bugs" in the offices of a milling company in Minneapolis shortly following a fumigation which netted 150 dead mice and presumably numerous rats, as well. The fumigator was convinced that the mites originated in wheat samples in the laboratories and not even the demonstration of mammalian red corpuscles in them changed his viewpoint. The mites soon disappeared.

Three years ago, occupants of a St. Paul apartment house complained bitterly regarding attacks of mites which were likewise determined as *Liponyssus bacoti.* The house was rat-infested and near a dump which harbored many rats.

In late January of this year the State Department of Health submitted specimens reported as causing "peculiar, itching, macular skin eruptions" on employees of a local department store at Pipestone, in the southwestern part of Minnesota and within a few days similar specimens were received from Blue Earth, Minn., where they had invaded an apartment in a remodeled old store building. A unit of the city heating plant had been installed and as there were many rats about the building and adjacent stores the set-up for mite infestation was perfect.

In addition to these cases, there have been reported several in which it seemed highly probable that *Liponyssus bacoti* was concerned although no specimens were received. The fowl mite, *L. sylviarum,* is not uncommon in Minnesota, but while it may cause considerable annoyance to handlers of live poultry, I have not encountered any cases of severe dermatitis in man, due to its attacks.

I am obligated to Dr. H. E. Ewing who examined specimens of the mites from Pipestone and Blue Earth and pronounced them typical *L. bacoti.*—William A. Riley, *University of Minnesota.*

THE SNOWSHOE HARE A NEW HOST OF *DERMATOXYS VELIGERA* AND *NEMATODIRUS LEPORIS*

The cottontail rabbit, *Sylvilagus floridanus,* is commonly reported as the host of the nematodes *Dermatoxys veligera* and *Nematodirus leporis.* Neither one of these species has hitherto been recorded for the snowshoe hare, *Lepus americanus.*

At Lake Alexander and Lake Mille Lacs, which lie within the southern boundary of the Canadian Zone in central Minnesota, the ranges of the cottontail and the snowshoe hare overlap. Here one might expect to find the parasites of one host infecting the other.

A snowshoe hare, *L. a. phaeonotus,* collected on January 23, 1933, south of Lake Mille Lacs, was found by Dr. F. G. Wallace to harbor 10 specimens of *Dermatoxys veligera.* Another hare, collected on June 9, 1934, by the Minnesota Wildlife Disease Investigation at Lake Alexander, was found by the writer to be infected with 42 *Dermatoxys veligera* and 33 specimens of *Nematodirus leporis.*—Arnold B. Erickson. *Paper No. 1812 Scientific Journal Series Minnesota Agricultural Experiment Station, St. Paul. In cooperation with the Minnesota Conservation Department, Division of Game and Fish.*

AMERICAN SOCIETY OF PARASITOLOGISTS

PRELIMINARY ANNOUNCEMENT OF THE SIXTEENTH ANNUAL MEETING

MONDAY, TUESDAY AND WEDNESDAY, DECEMBER 30 AND 31, 1940, AND JANUARY 1, 1941

PHILADELPHIA, PENNSYLVANIA

Pursuant to the action taken at the fifteenth annual meeting in Columbus, Ohio, the American Society of Parasitologists will convene for a 3-day program in conjunction with the meeting of the American Association for the Advancement of Science in Philadelphia, Pennsylvania. The Bellevue-Stratford Hotel has been designated as headquarters for the Society, and the program sessions will be held in the College of Physicians Building, which is located midway between the downtown area and the campus of the University of Pennsylvania.

The main features of the program are arranged for the second day, Tuesday, December 31st. At the conclusion of the morning session, the presidential address will be delivered by Dr. David H. Wenrich on the subject "The Morphology of Some Protozoan Parasites in Relation to Microtechnique." The annual Parasitologists' Luncheon and annual business meeting of the Society will be held at noon following the presidential address. The demonstration program and tea will be held in the afternoon. Since the regular sessions for the reading of papers are each year becoming more and more crowded, it is hoped that as many papers as possible will be presented by demonstration.

The Symposium on Malaria, which has been arranged under the auspices of Section N of the American Association for the Advancement of Science with the American Society of Parasitologists as one of the joint sponsors, will be held on the same days and in the same building as the meetings of the Parasitologists. Depending upon the time available, one or more of the parasitology sessions will be scheduled to meet jointly with the symposium.

The call for papers has already been sent to members of the Society. In preparing abstracts, members are requested to consult the styling used in the December, 1939, Supplement of the Journal in order that editing may be kept at a minimum. Members are reminded that all abstracts for the program must be in the office of the Secretary not later than Friday, October 25, 1940.

O. R. McCoy, *Secretary*